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JESSE HOWARD MUNCIE, 1890-1954

D. J. deZeeuw and W. J. Hooker

Jesse Howard Muncie, Professor of Botany and Plant Pathology of Michigan State College, died suddenly of a heart attack at his summer home on Elk Lake, Grand Traverse County, Michigan, on July 4, 1954. His death preceded anticipated retirement by less than a year.

Dr. Munice was born the second son of Sarah Varley and William R. Muncie, July 3, 1890, at Middlebury, Indiana. His early years as the son of a minister were spent in a number of communities in central and southern Illinois. He was graduated from high school in Paris, Illinois, in 1908 and obtained a B.A. degree in Botany from Wabash College in 1912. While there he was influenced by the example of H. H. Whetzel and Charles Chupp, his predecessors at Wabash, to begin graduate study at Cornell University in Plant Pathology. In 1917 he was granted the M.A. degree from Cornell University following investigation of "The effect of seed treatment upon susceptibility to disease in bean plants". He served as Assistant Botanist with the Ohio Agricultural Experiment Station at Wooster, Ohio, working on diseases of vegetable, field, and greenhouse crops from April to October, 1913. From 1913 to 1917, he worked primarily on diseases of beans at the Michigan Agricultural Experiment Station, although some attention was given to diseases of potatoes and sugar beets. He spent a year during World War I as inspector with the Plant Disease Survey, United States Department of Agriculture, at Washington, D. C.

From 1918 to 1921 he was engaged as Assistant Plant Pathologist in tomato disease research for the Pennsylvania State College field laboratory at Girard.

J. H. Muncie entered Iowa State College in 1921 as Assistant Pathologist and began work toward the Ph.D., which he received in 1925 after submitting the thesis "A study of crown gall caused by *Bacterium tumefaciens* on rosaceous hosts". He continued his research on crown gall at Iowa as Research Fellow and Agent, United States Department of Agriculture, until 1929. He was state leader of the Barberry Eradication Program, 1922-1924.

In 1929, he returned to Michigan Agricultural College as Research Associate and as Extension Associate Professor in the Department of Botany and Plant Pathology. He was promoted to Professor in 1943.

During his 25 years in research and extension at Michigan State College Dr. Muncie's responsibilities were extensive. His contacts among growers, county agents, and members of agricultural industry were many and cordial, and his advice was sought by many after he left the Extension Service. For the last 10 years his research was confined largely to potato pathology, but he maintained his broad interest in the diseases of vegetables and cereals with which he had worked.

JESSE HOWARD MUNCIE, 1890-1954

Dr. Muncie was a member of the American Phytopathological Society, the Potato Association of America, Gamma Sigma Delta, Epsilon Sigma Phi, and Sigma Xi. He served as vice-president of the Potato Association of America in 1952 and as president in 1953. In 1952, as chairman of the Sustaining Membership Committee, he made continued effective operation of the association possible by virtue of his outstanding leadership and generous service.

He was married in September, 1913, to Helen M. Baber whose charm and unselfish consideration for others did much to endear the family to their friends and college associates. Besides his wife, he is survived by 2 sons, James and Robert, and by 2 daughters, Louise Rochm and Margaret Evatt. Helen Fereday, another daughter, preceded him in death in 1946.

Dr. Muncie was the author or co-author of more than 60 professional papers on diseases of potatoes, beans, sugar beets, tomatoes, cucumbers, nursery stocks, and cereals.

The loss of Dr. Muncie's enthusiasm and capable work in potato pathology will be deeply felt by his colleagues in the department, on the campus at Michigan State College, and in the professional societies of which he was a member.

PHYTOPATHOLOGY for September (45: 467-522) was issued September 15, 1955

HISTOLOGICAL STUDIES ON THE ROLE OF PECTIC ENZYMES IN THE DEVELOPMENT OF FUSARIUM WILT SYMPTOMS IN TOMATO [1]

C. F. Pierson, S. S. Gothoskar, J. C. Walker, and M. A. Stahmann

SUMMARY

The development of vascular plugs was traced in tomato cuttings that had been treated with a culture filtrate from the tomato wilt Fusarium (*Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans.). The filtrate contained pectin-methyl-esterase and depolymerase. Similar studies were made with cuttings treated with a commercial pectic enzyme preparation containing pectin-methyl-esterase and polygalacturonase. Parrafin sections prepared from infected plants that were beginning to wilt and from treated and untreated plants at 12, 24, 36, and 48 hours after the beginning of the experiment were stained with ruthenium red. In the treated plants, vascular plugs developed progressively, starting with small clumps or strands at 12 hours, and ending with almost complete plugging at 48 hours. Concomitantly, increasingly large clear areas developed in the middle lamellae. Vascular discoloration was visible microscopically at 24 hours, and macroscopically at 48 hours. At the latter time, wilt symptoms were well developed in the treated plants. In the diseased plants, vascular plugs of the same type were also found. Cuttings treated with a pectin-methyl-esterase from tobacco exhibited neither vascular discoloration nor plugging at the end of 48 hours.

Recent investigations concerning the mechanism of wilting in tomato plants infected with *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans. have pointed to vascular plugging as a major factor in impeding the translocation of water and mineral salts in the host. Ludwig (8) reported the presence of hyaline plugs in fresh sections of infected tomato stems, but he was unable to demonstrate them in paraffin sections. He concluded that the material was probably a pectic gel which was lost in the dehydration process. Davis (2) found a β-glucosidase in the sap of infected plants but not in sap of noninfected plants. He postulated that the fungal β-glycosidase broke down the β-glycosides in the plant thereby producing phenolic compounds that caused vascular discoloration. Plugging could be due to the accumulation of these high molecular weight fragments. Scheffer and Walker (9) demonstrated vessel blocking in both infected plants and in plants treated with fungus filtrate by comparing the uptake of dyes by living plants, but they did not show the actual plugs. Gothoskar et al (4, 5) were able to duplicate Fusarium wilt symptoms in tomato by using fungus filtrates or pectic enzymes, and they postulated the formation of plugs by the release of viscous pectic materials into the vessels. By the use of models, Waggoner and Dimond (10) studied the reduction of water flow by mycelium in tracheal elements. They concluded that the mycelium was a contributing factor, but that it could not be the total cause of impeded water flow.

Since it has been shown that vascular plugging and wilt can be produced by pectic enzymes secreted by the tomato Fusarium, a series of experiments was carried out to follow by histological study the development of these plugs in tomato plants treated with fungus filtrates and with commercial enzyme preparations.

MATERIALS AND METHODS.—Preliminary experiments showed that a commercial enzyme, Pectinase (Nutritional Biochemicals Corp.), and the culture filtrate from the tomato Fusarium produce identical wilt symptoms in tomato. Enzyme assays indicated that Pectinase and Fusarium culture filtrates each possess both pectin-methyl esterase (PME) and enzymes capable of splitting the pectin chain. Whereas the Fusarium enzyme has been shown to be a depolymerase (5), the commercial pectinase possesses all the enzymes necessary to degrade the pectin molecule to monogalacturonic acid. Thus Ayers et al (1) and Dingle et al (3) have analyzed mold enzyme preparations and have concluded that their crude polygalacturonase preparation contained at least 3 pectin-degrading enzymes. Enzyme preparations from fungus filtrates and from a commercial source were used in parallel experiments to determine if there were any differences in action within the host. A 0.5 per cent solution was prepared from the commercial enzyme, and the fungus filtrate was used full strength. Both preparations were divided into equal parts. One part of each was autoclaved at 15 lb. pressure for 90 minutes, whereas the other remained unheated. All preparations were then filtered through a Seitz filter to remove precipitates in the first case and contaminating organisms in the other.

Bonny Best tomato plants of uniform size were removed from the pots, and the sand was washed from the roots. The lower 15 cm of each plant was placed in 95 per cent ethyl alcohol for 2 minutes to minimize surface contamination, and cuttings were made in sterile tap water. The cuttings were then placed in sterile flasks containing 50 ml of either heated or unheated enzyme solution, and cotton plugs were inserted

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into the necks of the flasks to eliminate air-borne contaminants. Tissue samples were collected at 12, 24, 36, and 48 hours after the beginning of treatment. These tissues, along with those of check plants that had been placed in distilled water and those of infected plants that were beginning to exhibit wilt symptoms, were fixed in FAA (formalin 4 per cent, acetic acid 3 per cent, ethyl alcohol 43 per cent, distilled water 50 per cent), dehydrated in the tertiary-butyl alcohol series, and embedded in paraffin. Sections were cut

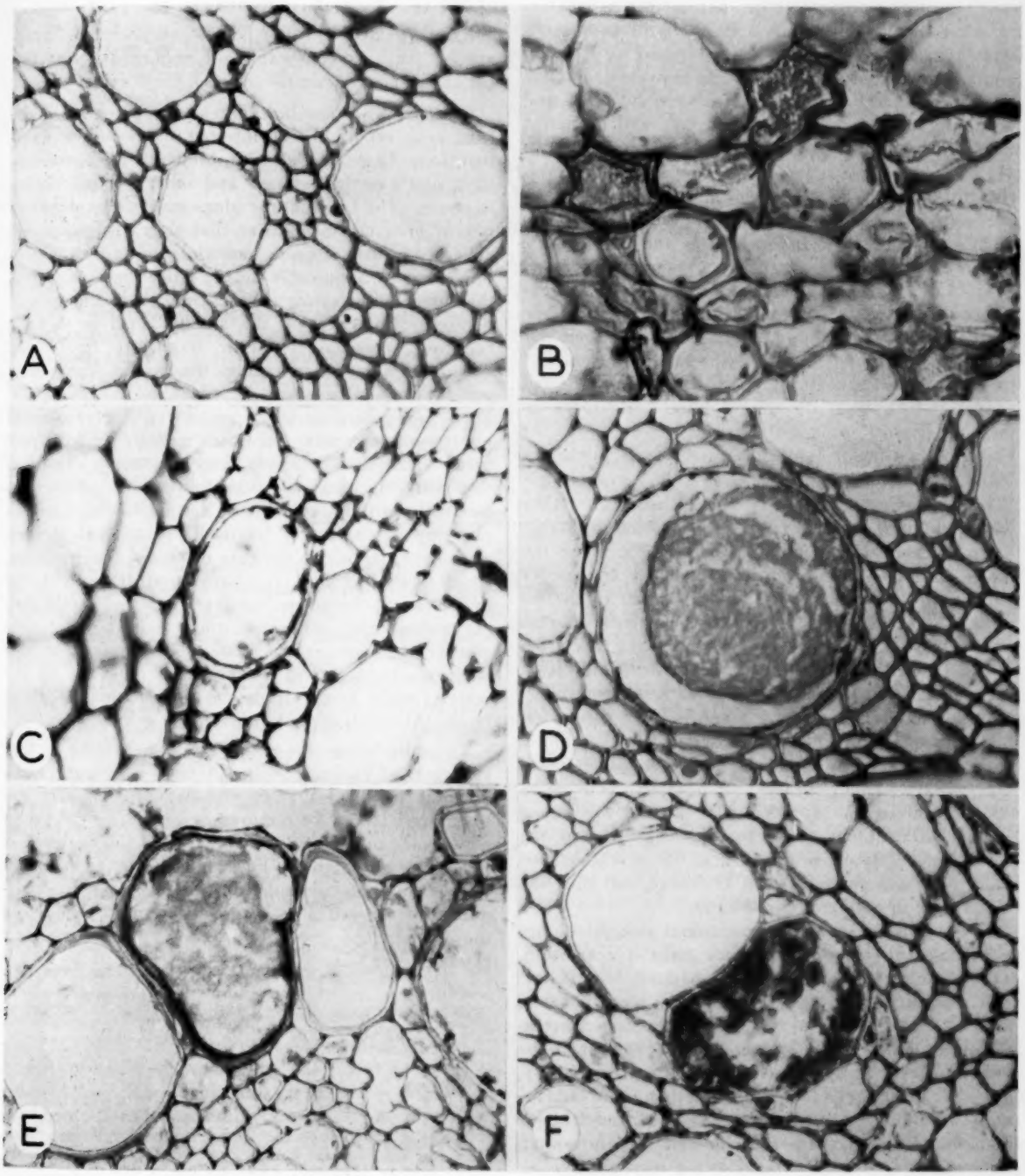

FIG. 1. Photomicrographs of tomato stem tissue stained with ruthenium red. A) Healthy tomato stem. B) Infected stem showing plugs in some of the vessels. C) Stem 12 hours after treatment with enzyme, showing deeply staining material along the inner margin of the vessel and heavy staining of lamellae of xylem parenchyma. D-F) Stem 24, 36, and 48 hours, respectively, after treatment with enzyme, showing progressive development of vascular plugs.

at 12μ on a sliding microtome and stained for 60 minutes in a 1:5000 aqueous solution of ruthenium red.

For use in experiments on the role of pectin-methyl-esterase in the disease syndrome, a preparation of this enzyme was obtained from tobacco leaves by the method of Gupta (6). This enzyme preparation had high pectin-methyl-esterase activity, but neither polygalacturonase nor deploymerase activity could be demonstrated in it. Tomato cuttings prepared as outlined above were placed in heated and in unheated solutions of this enzyme. Freehand sections were cut at 24 and 48 hours, stained with ruthenium red, and examined microscopically for vascular browning and plugging.

Experimental results.—The inner walls of the vessels of check plants were clean and smooth in all experiments. The walls themselves did not stain, but the middle lamellae between the vessels and between the vessels and the xylem parenchyma stained a deep red (Fig. 1, A.). There were also deep-staining areas where the corners of the vessels and the xylem parenchyma cells met. Neither vascular discoloration nor plugging was found in any of the material from cuttings treated with heated preparations. The unheated preparations of both the fungus filtrate and the commercial enzyme produced identical results in the plants, hence the following discussion will suffice for both.

In sections taken from cuttings 12 hours after they were placed in the filtrate or enzyme solution, a thin layer of deep-staining material was visible along the inner margins of the vessel walls, and strands or clumps of the same material were present in the lumina of affected vessels (Fig. 1, C). Small clear areas were visible between the vessels, and there was a decrease in staining intensity at the corners, indicating that some of the pectic materials had been removed.

In sections taken after 24 hours, isolated vessels exhibited the same deep-staining layer along their inner margins. In addition, however, there were large, granular, faintly staining masses that occupied as much as 70 per cent of the cross-sectional area of the lumen (Fig. 1, D). Clear areas between cells and at the corners were more distinct than at 12 hours. At this time there was some vascular browning, but this was discernible in sectioned material only.

In the 36-hour sections, the material along the inner margins of the vessel walls took on a deeper stain. The granular material within the lumen was denser and stained more deeply than that in sections taken after 24 hours. Many of the smaller vessels were completely occluded, while as much as 75 per cent of the lumen of some of the larger vessels was filled (Fig. 1, E). The clear areas between the cells and at the corners were much more distinct than in the 24-hour sections. The same was true for cell wall browning, although this was still not visible macroscopically.

After 48 hours, wilting symptoms had begun to appear, and vascular discoloration was quite distinct macroscopically. The vascular plugs were larger, denser, and more numerous than at 36 hours, and the clear areas between the cells were more extensive (Fig. 1, F).

Vessels of infected plants exhibited the same type of plugs as those in enzyme-treated plants (Fig. 1, B). There was also vascular occlusion by hyphae, but seldom more than 50 per cent of the cross-sectional area of any vessel was thus affected.

Plants treated with the pectin-methyl-esterase preparation from tobacco exhibited neither vascular discoloration nor plugging.

Discussion.—Vascular plugging, apparently of the same type, occurs in both infected plants and in plants that have been treated with preparations containing both pectin-methyl-esterase and other pectin-splitting enzymes. The fact that the plugs stain with ruthenium red in both cases indicates that they are composed, at least in part, of pectic materials. The pectin-splitting enzymes apparently diffuse from the vessels into the surrounding xylem parenchyma where they cleave the pectic materials in the middle lamellae to form relatively large soluble pectic fragments. Following this, the fragments move into the vessels where they gel to form plugs that impede the flow of water. Either polygalacturonase or pectin depolymerase could produce these results, since both initially split the pectin molecule into relatively large fragments. The fact that polygalacturonase ultimately splits pectin into monogalacturonic acid does not preclude vascular plugging by the larger fragments prior to their complete degradation. In the case of pectin depolymerase, which Gothoskar et al. (5) have shown to be produced by the tomato Fusarium, the degradation of pectin does not proceed to monogalacturonic acid but to compounds that are intermediates between pectin and monogalacturonic acid. Hence, the plugging of the vessels could result from the aggregation of these larger intermediate molecules into a gel.

The role of pectin-methyl-esterase in the disease syndrome of Fusarium wilt of tomato is obscure, since alone it produces neither vascular discoloration nor plugging. It may be required to de-esterify the pectin prior to the action of depolymerase. If this is the case, the depolymerase in the Fusarium filtrate may then act more rapidly and resemble in this respect a true polygalacturonase, which is known to act more rapidly on the de-esterified molecule than on the completely esterified one (7).

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A PENICILLIUM CROWN ROT OF ASPARAGUS [1]

J. D. Menzies [2]

A previously undescribed crown and root rot of asparagus has caused damage to overwintering asparagus plants in seedling nurseries in the Yakima Valley, Washington. The disease results from infection by *Penicillium martensii* Biourge following winter injury. The fungus is parasitic when established in injured tissue but does not attack uninjured plants. Evidence indicates that the fungus is widely established in both the older irrigated areas and in uncultivated land. The damage to crowns and storage roots occurs in late winter and early spring.

Control recommendations include hilling of plants in the fall to prevent freezing injury, careful roguing of infected plants during transplanting, and care in handling transplants to prevent unnecessary injury. Fungicidal treatment to protect crowns at transplanting time is of little value.

A previously unreported crown rot of seedling asparagus (*Asparagus officinalis* L.) has become prevalent during the past 10 years in the Yakima Valley of Washington. The damage occurs during winter and is revealed when the seedling plants are dug in the spring for transplanting. This report describes experiments showing that the disease is caused by the parasitic activity of the fungus *Penicillium martensii* Biourge following winter injury to the crowns.

HISTORY AND DISTRIBUTION.—This disease first attracted attention in the spring of 1946. Unusually large numbers of seedlings were grown in the following 3 years during which the presence of the "blue mold" was reported throughout the Yakima Valley. Although the records of the Washington Plant Disease Survey contain no reference to this disease prior to 1946, old-time growers claim to have observed it over 20 years ago when asparagus was a new crop in the area. Apparently it is an established disease that escaped notice until the war-time stimulus for increasing asparagus acreage brought it into prominence.

Species of *Penicillium* often have been reported as the cause of market diseases of harvested asparagus shoots, but previous records of Penicillium crown and root rots are rare. According to Dr. Freeman Weiss, records of the Plant Diseases Survey contain only 2 other references to such a disease, 1 from Oregon and 1 from California. No details accompany these notations nor do the State Experiment Stations in either state have further information. This would indicate that these fragmentary reports were made some years ago and probably did not represent serious disease problems.

ECONOMIC IMPORTANCE.—Penicillium rot is not an important problem in established cutting beds. The greatest loss results from planting seedlings so badly infected that they fail to survive, thus resulting in poor stands in the permanent beds. Experience has been that the replanting of missing hills is expensive in hand labor and is usually unsatisfactory.

This disease has caused occasional financial loss to growers of seedlings for sale. In the spring of 1947, a survey of these growers showed that some blue mold was present in nurseries throughout the irrigated areas of south central Washington. Only a single 10-acre nursery was found in which no blue mold occurred. In 16 other nurseries examined, infection ranged from 1 per cent to 47 per cent of the plants and averaged 17 per cent for the entire group.

SYMPTOMS.—Penicillium rot of asparagus is readily recognized by the typical bright blue masses of mycelium and spores produced on the diseased tissue. On freshly dug crowns the blue-mold growth is so conspicuous that affected plants can be readily sorted out.

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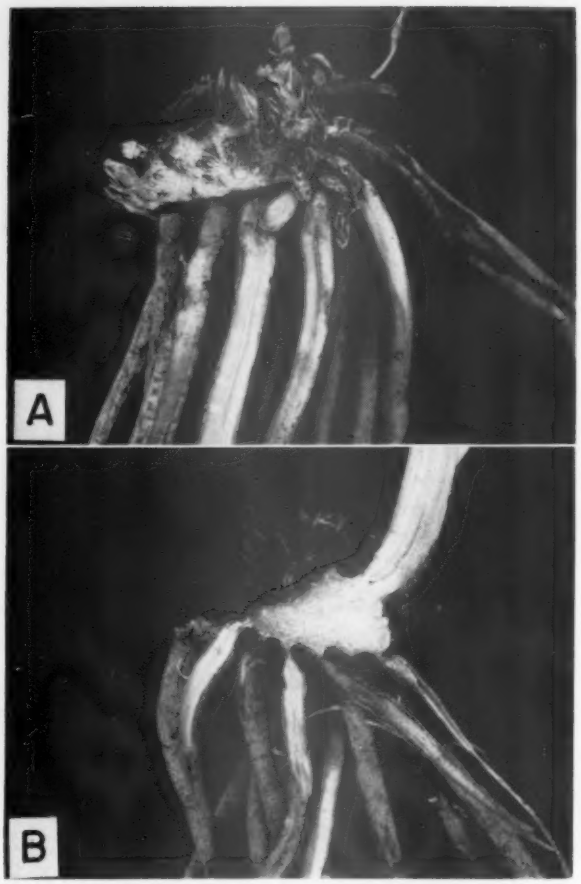

FIG. 1. Penicillium crown rot of asparagus. A) Naturally infected seedling plant cut to show necrotic crown tissue and infected storage roots arising from this portion of the crown. Discoloration of stele is evident in 1 sectioned root. B) Healthy crown of same age.

Usually the site of infection is the dormant crown buds and adjacent storage roots. As the crown grows laterally from the seed location it becomes progressively thicker, the buds become larger, and the storage roots heavier (Fig. 1, B). The rot is located typically at the older, thinner portion of the crown. Infection may be confined to the buds and crown tissue or may involve the storage roots as well. The rotted area frequently extends from the thin edge of the crown into the younger tissues as a dry pocket rot and is at first red to orange in color. Later it changes to a grey hollow type of lesion (Fig. 1, A).

Whenever the infection extends into the fleshy root system it can be detected externally by the dull brown appearance of the invaded root. Rot in these roots commences as a water soaking of the cortical tissue which soon becomes necrotic. The cortical cells separate into a mealy mass that disintegrates, leaving a dry collapsed root. Very often a reddish streaking of the central stele occurs in association with the cortical rot. This vascular discoloration usually does not extend more than a few mm beyond the evident lesion but has occasionally been found far beyond this point. In such cases the pathogen can be isolated from the discolored stele. This symptom is also characteristic of Fusarium rot of asparagus described by Cohn and Heald.[3] That the Penicillium and the Fusarium may produce identical symptoms in the stele was demonstrated in parallel inoculations with the 2 organisms. The Fusarium, however, does not produce the cortical rot.

Secondary infections commonly occur at various points on the storage roots after the plants have been dug and replanted. Infection takes place at injuries caused in digging, separating, loading, and replanting the crowns. These infections are very frequent, but they seldom are observed since the only aboveground indication of injury is less vigorous shoot growth. Storage-root infections do not spread far along the root but almost always cause a girdling at the point of infection. The young plant thus loses the benefits of the stored food in the root beyond the point of injury, resulting in delayed growth. This early stunting is overcome as new feeder roots develop.

THE CAUSAL FUNGUS.—A single species of *Penicillium* was consistently isolated from sporulating pustules and from infected tissue of crowns, buds, and roots. In some instances, *Fusarium oxysporum* (Schlecht.) Snyd. & Hans. also could be isolated from the discolored margins of crown lesions or from vascular tissue of the storage roots. It was at first considered probable that the Fusarium was the primary parasite. Inoculations and reisolation tests with both organisms, however, established the fact that the Penicillium could invade and rot asparagus tissue in the absence of the Fusarium.

Cultures of the causal organism were sent to Dr. Kenneth Raper[4] at the Northern Regional Research Laboratory, Peoria, Illinois. He identified it as *P. martensii* Biourge and commented as follows: "The culture is of particular interest to us because of its source for we have not previously known of this species or a member of the *P. cyclopium* series to which it belongs having been isolated as a cause of rot in asparagus. *P. cyclopium* has been implicated upon occasion in the decay of bulbs such as *Lilium* and other liliaceous plants."

This fungus is evidently widely distributed in the soil because it can almost always be found at injured areas on the roots of new transplants, regardless of the nursery source or the field in which they are planted. High percentages of infection have been noted in seedlings grown on land freshly cleared from sagebrush. For example, of 5 nurseries in which asparagus was grown in 1946 on previously uncultivated

[3] Cohn, S. I., and F. D. Heald. 1941. A wilt and root rot of asparagus caused by Fusarium oxysporum Schlecht. U. S. Dept. Agr. Pl. Dis. Rptr. 25: 503-509.

[4] Now Professor of Bacteriology and Botany, Bacteriology Department, University of Wisconsin, Madison.

land, 2 showed a trace of blue mold while in the others 23, 45, and 46 per cent of the plants were infected. In numerous tests it was found that artificial inoculation was unnecessary, since infection resulted naturally in a high percentage of cases where roots were variously injured and placed in unsterilized field soil.

INOCULATION EXPERIMENTS.—A series of inoculation experiments showed that *P. martensii* is a wound parasite, probably incapable of causing direct infection through uninjured root or crown tissue.

In 1 test, healthy asparagus crowns were divided into uniform lots and inoculated by various means. Storage roots injured by knife cuts or by slight crushing invariably became infected when dipped in spore suspension or inoculated by mycelial transplants. When roots were inoculated by needle punctures under spore suspension, infection was obtained in 23 of 30 inoculations. Needle punctures into crowns and buds followed by dipping in spore suspension were ineffective. When portions of the crown were scorched by a flame, however, Penicillium rapidly invaded the injured area and initiated a crown rot typical of that occurring in the field. No infections occurred on uninjured tissue in any of these tests.

Since these tests indicated that bud and crown infection was dependent upon previous injury, it seemed likely that freezing injury during the winter might be an important factor. That such injury predisposed crowns to infection was demonstrated by artificial freezing of crowns. Healthy young crowns were placed in containers deep enough to allow the buds to be covered with ½ in. of damp sand. The containers were then placed in a holding cabinet maintaining a temperature of 5° F for various lengths of time. All plants exposed for 7.5 hours or longer were partially to completely frozen. Although these plants were not artificially inoculated there was sufficient contamination of the plants to produce uniform Penicillium infection in injured tissue. The fungus invaded the injured buds and produced an internal crown lesion typical of the disease in the field. There was no freezing injury nor penicillium crown rot on any of the plants held in the low temperature cabinet for shorter periods of time.

PREDISPOSING FACTORS.—The preceding demonstration that freezing injury can lead to Penicillium crown rot is in agreement with certain observations on disease occurrence in the field. A survey in the spring of 1947 showed a strong tendency for damage to be worse in early-seeded nurseries. In such fields plants are larger and more mature by winter than plants in late-seeded fields. It is characteristic of asparagus seedlings to heave the soil along the row so that by the end of the season fissures are formed around the stem and between plants, often exposing the crown buds. This condition is more pronounced with large plants than with small ones. Thus, the larger the plant the greater will be the amount of crown tissue likely to be injured by low temperature during the winter.

All the asparagus in the Yakima Valley is irrigated, almost entirely by the furrow method. On steep slopes, erosion sometimes deposits a layer of silt around plants at the lower end of the rows. Numerous instances were found where this had occurred, and in these cases blue-mold rot was absent in the silted areas of the field but common farther up the slope. It is believed that the extra soil covering was sufficient to prevent winter injury.

Thin stands of evenly spaced plants were found to be less severely affected by Penicillium rot than thick stands. This may also be explained on the basis of less soil cracking.

EXPERIMENTS ON CONTROL.—Since injury to crown tissue, presumably from freezing, is necessary before crown rot occurs, the most obvious control procedure is to prevent this injury. Only 1 attempt has been made to test the value of hilling, mulching, or other means of cold protection in preventing blue-mold rot. This test failed to provide positive information because the winter of the test was mild and no rot developed in any of the treatments. Growers generally have been advised to practice some type of soil cultivation late in the fall to provide better soil cover for the seedlings. Those who have followed this practice report good results.

Asparagus crowns are unavoidably injured to some extent during the transplanting operation. Since infection by *P. martensii* at points of injury is very common, it was thought desirable to test the value of fungicidal dips both as protectants and as eradicants. Experiments were conducted in cooperation with the California Packing Corporation on their asparagus plantings near Toppenish, Washington. In 1 test a number of different fungicides were used on carefully selected healthy and diseased crowns. One hundred crowns of each class were used for each of 4 replicates of 6 fungicidal dips. Crowns were given a momentary dip sufficient to wet them thoroughly. They were planted at once in a commercial planting operation. Counts of stand and number of shoots per plot were made during the season and total top growth estimated by sampling at the end of the season.

In spite of the apparent benefits from the fungicides (Table 1), variability was so high that the differences just failed to be statistically significant at the 5 per cent level. By the end of the season the check plants had recovered or caught up to the treated plants, and total top growth was not significantly different. There was a decided difference, however, in both stand and growth between the healthy and diseased groups.

In a second experiment carried out the following year, 4 fungicides were tested at 4 concentrations. Each treatment involved 50 plants for each of 5 replicates arranged in a Latin square design for each fungicide. The freshly dug crowns were carefully sorted and sized and placed in open-mesh onion sacks for dipping. A standardized dipping procedure of 30 seconds duration was followed. Treated plants were kept in moist storage for 48 hours before planting. Three months after planting, stand counts were taken.

TABLE 1.—*Stand and growth of healthy and infected asparagus transplants following dip treatments with various fungicides*

Treatment	Dosage[a]	Shoots per 100-ft. row		Yield of tops[b]	
		Healthy	Diseased	Healthy	Diseased
		No.	No.	g	g
None		78	22	749	677
Mercuric chloride	1:1000	129	46	903	588
Chlorox	1:1000	133	63	1011	522
Fermate	1 lb./10 gal.	112	64	1058	507
Thiosan	1 lb./10 gal.	121	75	1044	547
Phygon wettable	1 lb./10 gal.	119	78	1194	573
Semesan Bel	1 lb./10 gal.	135	81	936	774

[a] Dosage is based on the formulated product. Active ingredients of the fungicides are as follows: Chlorox, 5.25 per cent sodium hypochlorite; Fermate, 76 per cent ferric dimethyldithiocarbamate; Thiosan, 50 per cent bis(dimethylthiocarbamoyl) disulfide; Phygon, 50 per cent 2,3-dichloro-1,4-napthoquinone; Semesan Bel, 3.2 per cent 2-chloro-4-(hydroxymercuri)phenol and isomers plus 12 per cent 4-(hydroxymercuri)-2-nitrophenol and isomers.

[b] Dry weight from 20-ft. row.

None of these treatments were effective in improving the stand of plants (Table 2). The higher rates of the New Improved Ceresan were injurious.

It was concluded that if crowns were graded to eliminate the obvious infections, the fungicidal dips were not likely to improve the percentage survival even though they may have given the transplants a better start. In other words, plants with no established crown infection could be expected to grow without protection in spite of infection of storage roots after transplanting. The young transplant depends upon the storage roots for only a short time while shoots and new roots are being established. Destruction of these storage roots delays this process but by the time commercial harvesting begins 2 years later the difference is not detectable.

The crown continues to renew itself as the older portion sloughs away. A crown infection must be active enough to destroy this tissue faster than it grows, or the plants "grow away" from the disease. The diseased crowns in the first experiment were either killed in the first season by the blue-mold or completely recovered. It is estimated that less than ⅓ of these diseased plants failed to recover.

TABLE 2.—*Survival of transplanted asparagus crowns following dip treatments with fungicides at various concentrations*

Dosage[a] lb./100 gal.	Percentage stand 3 months after treatment with			
	Phygon	Spergon	N.I. Ceresan	Semesan Bel
0	78.8	74.8	74.4	78.8
1.25	77.6	78.8	71.2	77.6
2.5	81.6	80.0	56.4	81.6
5.0	79.2	78.0	70.8	79.2
10.0	86.4	85.2	30.4	86.2

[a] Dosage is based on the formulated product. Active ingredients of Phygon and Semesan Bel are given in Table 1. Spergon is 96 per cent tetrachloro-*p*-benzoquinone and New Improved Ceresan is 5 per cent ethylmercury phosphate.

CONTROL RECOMMENDATIONS.—Several control measures are suggested by the experiments and observations on this disease.

1) *Protect seedlings from winter injury.*—This is most easily done by slight hilling late in the fall. It may be necessary to allow a slightly wider row spacing than normal to do this without exposing roots or crowns from the side.

2) *Grade crowns for blue-mold at digging time.*—Since the bright blue mycelial masses of the fungus are very obvious, rapid hand sorting is not difficult.

3) *Avoid excessive mechanical injury during harvesting and planting.*—These injuries provide infection sites. The fungus usually girdles storage roots at point of infection. Broken shoots delay emergence of plants, and this results in further weakening of the plants.

4) *Prevent drying-out of crowns after harvest.*—The drying of crowns between digging and replanting weakens them so that new growth is delayed. If the crown is infected the delay may result in complete rotting of the plant.

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RECOVERY OF WATER PIMPERNEL FROM CURLY TOP AND THE REACTION OF RECOVERED PLANTS TO REINOCULATION WITH DIFFERENT VIRUS STRAINS [1]

C. W. Bennett [2]

SUMMARY

Strains of curly-top virus of different degrees of virulence produced a wide range of severity of reaction on water pimpernel (*Samolus parviflorus*). Symptoms ranged from vein clearing, slight leaf rolling, and little or no stunting produced by the less virulent strains, to severe curling, distortion, yellowing, and necrosis of leaves and death of a part or all of the affected plant produced by the more virulent strains. In plants that survived the early effects of the disease, recovery from symptoms was complete or nearly so. Plants that recovered from the more virulent strains, however, were somewhat less vigorous than healthy plants.

No evidence was found that virus strains were attenuated or otherwise modified in recovered plants. Recovery was associated with a marked reduction of virus concentration.

Transmission of virulent strains from recovered plants by means of dodder, *Cuscuta californica*, resulted in the production of severe symptoms of disease in the inoculated plants, indicating that no protective principle was transported from recovered to healthy plants.

Added symptoms indicated that plants that have recovered from one virus strain are susceptible to infection with any strain more virulent than the one from which the plant recovered. It was not determined whether recovered plants are susceptible to infection with strains less virulent than the strain already present, but it was shown by means of a differential host that strain 1, which is about equal in virulence to strain 3, is superimposable on strain 3 although it produces no added symptoms.

Less virulent strains failed also to protect against injury by the more virulent strains. Plants that had recovered from strains of a medium to high degree of virulence, however, were sometimes protected against the full effects of strains of greater virulence.

Recovery of tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* Mill.) from curly top disease has been reported (3, 7, 8). Recovery nearly always follows infection of Turkish and certain other varieties of tobacco if the plants survive the early stages of the disease. *Nicotiana glutinosa* L. recovers nearly completely from some virus strains and less completely from others. Recovery is rare in commercial varieties of tomato under field conditions, but Wallace and Lesley (10) found recovery in a relatively high percentage of infected plants of Guasave-A tomato. Also a slight amount of recovery has been noted in sugar-beet plants infected with certain strains of the virus in early stages of growth. An appreciably greater amount of recovery occurs in sugar-beet plants infected with the Argentine curly top (2). Other species and varieties of plants infected with curly top have shown little or no recovery. The observation that water pimpernel (*Samolus parviflorus* Raf.), a previously unreported host plant of curly top, showed a high degree of recovery from the virus strains tested, therefore, seemed worth investigating. This paper reports some of the results obtained in studies of the recovery of this plant.

STRAINS OF VIRUS USED AND DEGREE OF RECOVERY.—Water pimpernel plants were inoculated with 14 strains or selections of curly-top virus. Seven of these, strains 1, 2, 3, 4, 6, 7, and 8, were described by Giddings (4, 5). Four selections, designated B, F, P, and T, from various sources and 3 selections carried by N. J. Giddings under the numbers 22C, 31, and 66 were included because they appeared to differ from each other and from described strains.

The reaction of water pimpernel to these strains varied with the virulence of the strain. The less virulent strains, including strains 2, 4, 7, 31, and F, produced vein clearing and vein swelling but little leaf rolling or dwarfing. Plants recovered in a relatively short time, showed faint symptoms or none after recovery, and did not show obvious relapse at any time.

Strains of medium severity, including 66, P, and T, produced marked leaf rolling and stunting in addition to the usual vein clearing and swelling. Under favorable conditions all infected plants recovered, and vegetative progeny showed little or no evidence of disease.

Virulent strains, including strains 1, 3, 6, and B, produced marked clearing and swelling of veins and rolling and dwarfing of leaves. Flower shoots were dwarfed and distorted, and growth of axillary buds was induced. Four to 6 weeks after inoculation, older leaves and many of the younger ones began to yellow and die. Much of the root system also died and sloughed off, and some of the plants died. Plants that survived sent out relatively normal shoots from the terminal and axillary buds. These shoots developed their own root systems. Usually all of the older parts of the plants eventually died. The recovered shoots

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grew fairly rapidly and developed into plants almost free of symptoms. In rare instances some of the plants from recovered shoots, after growing normally for a time, showed an obvious relapse, but recovery from the relapse was rapid and the plants were not seriously injured. Plants in different stages of recovery from virus B are shown in Figure 1.

Very virulent strains, such as 8 and 22C, produced still more severe symptoms, and many of the inoculated plants died without showing appreciable recovery. Plants that survived sent out slow-growing shoots from the crowns. Recovery was complete, or nearly so, from the vein-clearing, vein-swelling, and leaf-curling phases of the disease, but the plants remained small and the leaves were thick and leathery.

Recovered plants infected with each strain, except 8 and 22C, were increased by vegetative means for use in further tests. Under conditions of high humidity, roots were produced from the base of axillary shoots and from the nodes of the flowering stems. When planted separately, each shoot developed into a plant with a single terminal bud and a rosette of leaves. Many recovered plants gave 20 or more offshoots, and these in turn were multiplied after a period of growth. Plants of this type, usually of the second or later vegetative generations, were used for cross-protection and other tests with the different virus strains.

Tests of virulence of virus in recovered plants. —It is obvious that recovery of plants from the effects of virus infection may result from 1 or more of several factors: 1) The virus may become attenuated in the recovered plant and thus produce less injury; 2) a mixture of strains may be introduced in the original inoculum, and an attenuated component may become the dominant one; 3) the concentration of virus in the recovered plant may be reduced by the operation of some factor or factors to a level at which it no longer is able to produce symptoms comparable in severity with those produced by a maximum virus concentration; or 4) recovery may be due to other factors.

A series of tests was made to obtain evidence on whether the recovery of water pimpernel from curly top is due to either virus attenuation or differential multiplication of a virus of low virulence. In these tests, plants that had recovered were increased by separating rooted axillary shoots and by planting these singly in pots. Nonviruliferous beet leafhoppers were allowed to feed on each plant for 3 days and then transferred to noninfected plants of water pimpernel and sugar beet. As checks, equal numbers of plants were inoculated by means of beet leafhoppers direct from a sugar-beet plant carrying the same virus strain.

Symptoms produced by virus from recovered water pimpernel plants were typical of those of the strain involved in every instance. The symptoms did not differ from those produced following direct transfer of the same strain from sugar beet. Plants of water pimpernel inoculated from the 2 virus sources recovered at about the same rate and to about the same degree.

Strains 3 and B were held in water pimpernel for successive transfer tests. After each inoculated plant had recovered, the virus was transferred to a healthy plant of water pimpernel. After 9 such transfers over a period of 2 years, there was no evidence of change in virulence or other characteristics of either virus strain.

It was concluded from these results that the virus found in recovered plants of water pimpernel exists there in a degree of virulence equal to that main-

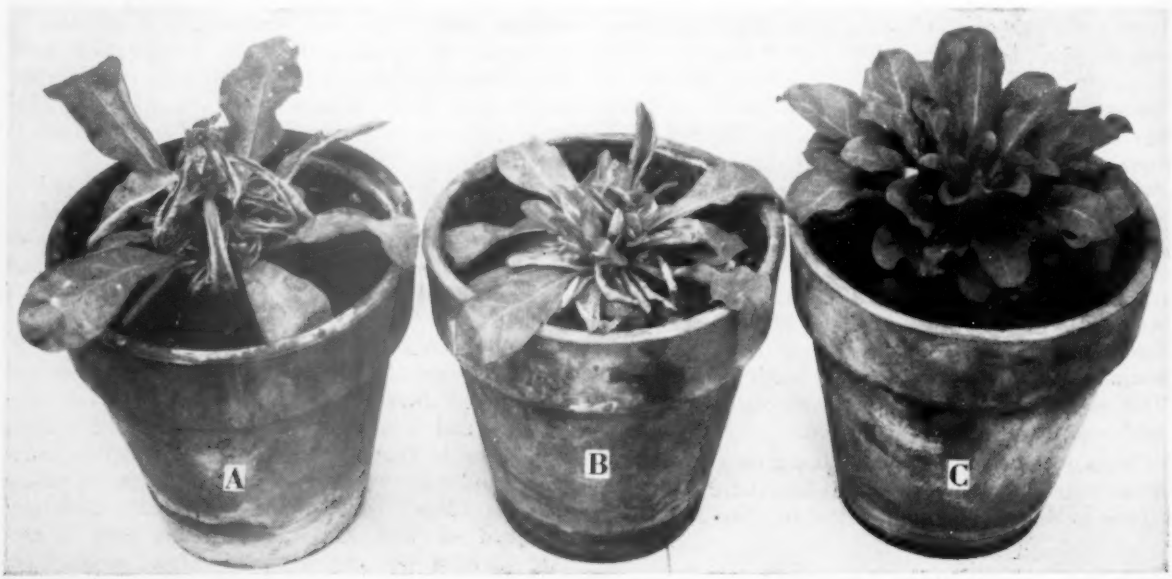

Fig. 1. Plants of water-pimpernel infected with curly-top virus strain B. A) Plant recently infected and showing leaf curling and dwarfing. B) Plant showing beginning stages of recovery by production of more or less normal shoots from axillary buds. C) Offshoot of fully recovered plant still carrying the curly-top virus but showing no symptoms of disease.

tained in sugar beet and that recovery is due to factors other than decrease in virulence of the virus or disproportionate increase of a less virulent strain. Any other explanation would involve postulation of a differential selection from the recovered plant of a virulent strain from a mixture of virulent and less virulent strains by the beet leafhopper, for which there is no evidence.

VIRUS CONCENTRATIONS IN RECOVERED AND NONRECOVERED PLANTS.—*Methods.*—Since curly-top virus is not transmissible by juice inoculation by the usual rubbing methods and is rarely transmitted by the needle-puncture method, determination of virus concentration is more difficult and somewhat less accurate than with certain mosaic viruses that are transmitted readily by juice inoculation. Methods are available, however, that yield results that give a concept of relative virus concentrations when differences are large. These methods, which utilize the beet leafhopper (*Circulifer tenellus* (Baker)), were used in tests of virus concentrations in paired plants. The plants of each pair in these tests were about the same size and age but one was in the recovery stage and showed no obvious symptoms and the other showed severe symptoms as a result of more recent infection. Three methods of test were employed.

1) *Method 1.*—Juice was pressed from equal numbers of the younger leaves from each plant of a pair selected for test. The juice formed a heavy precipitate almost immediately. This was thrown down by centrifugation, washed once with 50 per cent ethyl alcohol, and dried. The dry precipitate was resuspended in an aqueous solution of 0.01 M sodium citrate and 3 per cent sucrose equal in volume to that of the original juice. The solid matter was removed by centrifugation, and nonviruliferous beet leafhoppers were allowed to feed on the supernatant liquid through an animal membrane for 6 hours. The leafhoppers were then caged singly on seedling sugar-beet plants for 7 days. The number of infected plants indicated relative virus concentrations in the extracted juice of the respective plants and presumably also the relative virus concentration in the tissues of the plants from which the extracts were derived.

2) *Method 2.*—Nonviruliferous beet leafhoppers were allowed to feed for 24 hours on infected plants and then caged singly on seedling sugar-beet plants for 7 days. The resultant number of plants infected is assumed to be a measure of the relative amount of virus the leafhoppers were able to pick up from the respective plants in a 24-hour feeding period and, therefore, an indication of relative concentration of virus in the infected plants on which the leafhoppers fed.

3) *Method 3.*—Nonviruliferous beet leafhoppers were permitted to feed 3 days on diseased plants. They were then removed and chloroformed, and 40 leafhoppers from each lot were ground in a mortar and mixed with 2 ml of distilled water. The mixture was centrifuged and the supernatant liquid was mixed with 2 ml of 95 per cent ethyl alcohol. The resultant precipitate was removed by centrifugation, dried, and resuspended in 2 ml of an aqueous solution of 0.01 M sodium citrate and 3 per cent sucrose. The solids were removed by centrifugation and nonviruliferous leafhoppers allowed to feed on the supernatant liquid through an animal membrane for 6 hours. The leafhoppers were then caged singly on seedling sugar- beet plants for 7 days The number of plants infected is considered an indication of the relative virus concentration in the plant on which the ground leafhoppers had fed.

RESULTS.—Results of tests of 10 pairs of plants infected with strain B are shown in Table 1. More virus was obtained from the nonrecovered than from the recovered plant of each pair except in 1 instance. In the majority of pairs this difference was large. It is evident, however, that the recovered plant of some pairs yielded more virus by methods 1 and 2 than the nonrecovered plant of other pairs. This is believed to have resulted from the fact that these tests were conducted over a period of more than 6 months and never more than 2 pairs of plants and 1 method of test were used in 1 day. It is known that environment to which the plants are subjected for a few hours prior to testing influences the amount of virus recoverable by method 1. For instance, juice extracted from diseased plants subjected to high temperatures for 6 hours or more before testing often yields low amounts of virus. It seems probable that the virus recoverable by method 2 may be affected by the same or similar factors. The results shown in Table 1, however, indicate that regardless of what the influencing factors may have been they affected each member of a pair more or less proportionally.

By method 3, in which the time for leafhoppers to acquire virus from the diseased plant was extended over a period of 3 days, results were more uniform with respect to the different pairs. With this method, even the highest amount of virus from a recovered plant was less than the lowest amount from a nonrecovered plant. These results indicate very definitely that recovery is associated with a marked decrease in concentration of active virus.

TABLE 1. *Relative curly-top virus concentration in recovered and recently infected water-pimpernel plants*

Test method	Plant tested	Seedling sugar-beet plants infected from indicated diseased plants in test[a]									
		1	2	3	4	5	6	7	8	9	10
1	Recovered	4	20	6	7	20	13	7	3	13	20
	Nonrecovered	19	25	31	25	30	32	17	29	35	38
2	Recovered	24	8	5	8	33	11	12	27	4	5
	Nonrecovered	35	29	17	15	32	14	21	29	10	25
3	Recovered	9	10	18	21	24	16	22	21	15	14
	Nonrecovered	27	28	30	31	33	32	31	29	32	31

[a] Figures indicate plants infected of 40 inoculated by means of leafhoppers that previously had fed upon extracts of the infected water-pimpernel plants (Method 1), upon the infected plants (Method 2), or upon extracts of leafhoppers that had fed 3 days on the infected source plants (Method 3).

ATTEMPTS TO TRANSMIT A PROTECTIVE PRINCIPLE.—Wallace (8, 9) showed that a high degree of protection against injury by curly top is achieved in plants of tomato and tobacco by inoculating with scions from recovered tobacco or tomato plants. He suggested the possibility of the passage of a protective principle from the infected scion to the healthy stock along with the virus. So far, all attempts to determine whether this method of protection is effective with water pimpernel have met with failure because of the difficulty of obtaining union of tissues of this plant. Flower stalks of recovered plants were attached to similar stalks of healthy plants by various means, including approach grafts, but no union occurred. Also, attempts were made to graft parts of crown tissue but again no union occurred. Cut surfaces of this plant appear unable to form callus.

Other tests were made to determine whether a protective principle is passed from recovered to healthy plants by means of dodder. *Cuscuta californica* Choisy, growing on water-pimpernel plants recovered from strain B, was trained to healthy plants and allowed to become established before the connections with the diseased plants were severed. Of 40 plants inoculated in this manner, 27 became infected with curly-top virus. Symptoms were of the same degree of severity as those on check plants inoculated by means of the beet leafhopper, and plants infected by the 2 methods of inoculation recovered at about the same rate. No evidence, therefore, was found that a protective substance passes from recovered to healthy plants through a dodder stem connecting the 2 types of plants.

CROSS-PROTECTION TESTS.—Thus far, work with strains of curly-top virus has indicated a considerable range in degree of protection offered, depending on the virus strains and the host plants involved. Wallace (9) found that tomato plants once recovered from certain strains of curly-top virus remained highly resistant to injury by certain other strains but not to injury by all strains of the virus. No evidence was found that the recovered or protected plants were resistant to infection by a second strain.

In tests with 7 virus strains, Giddings (6) found little or no protection by 1 strain against either infection or injury by another strain of the virus in sugar beet. High degrees of recovery from curly top have not been observed in sugar beet.

Because of the marked recovery in water pimpernel and the fact that recovered plants may be propagated indefinitely by vegetative means, the resistance of plants recovered from various strains to infection and injury by other strains of the virus was tested. In these tests, inoculations were made with strains 1, 2, 3, 4, 6, 7, 31, 66, B, F, P, and T. After recovery, offshoots of recovered plants, or their progeny, were potted separately and reinoculated with other virus strains. Severity of symptoms induced by reinoculation of recovered plants was indicated by a grading system ranging in ascending order of severity from 1 to 5 in which grade 1 indicates vein clearing a slight amount of leaf rolling, and little dwarfing, and grade 5 indicates marked leaf rolling, vein clearing and vein swelling, yellowing and death of older leaves, and stunting and sometimes death of the plant. In assigning grades of severity to plants inoculated with a second strain, the symptoms given major emphasis and weight were vein clearing, vein swelling, leaf curling, leaf rolling, and leaf distortion and dwarfing. General dwarfing and slow growth were not considered except as they were evident in comparison with recovered check plants infected only with the strain of virus introduced first.

The results of these tests show that reinoculation with the strain of virus from which the plant had recovered did not result in the production of added symptoms (Table 2). Plants that recovered from the less virulent strains remained susceptible to infection with and to injury by more virulent strains. There was no evidence that plants infected with the milder strains were more resistant either to infection or to injury by more virulent strains than previously noninfected plants. This is illustrated in Figure 2, which

TABLE 2.—*Reaction of recovered water-pimpernel plants to reinoculation with different strains of the curly-top virus*

Strain of virus used in initial inoculation	Severity of symptoms after reinoculation with indicated strain or selection[a]													
	1	2	3	4	6	7	8	22C	31	66	B	F	P	T
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	4.5	0.0	5.0	0.0	4.7	0.0	5.0	5.0	0.0	0.0	3.7	0.0	3.1	3.1
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
4	4.8	0.0	5.0	0.0	5.0	0.0	5.0	5.0	0.0	0.0	4.2	0.0	3.3	3.2
6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
7	4.0	0.0	4.5	0.0	5.0	0.0	5.0	5.0	0.0	0.0	4.8	0.0	2.2	2.2
31	4.8	0.0	4.7	0.0	4.6	0.0	5.0	5.0	0.0	0.0	4.1	0.0	3.0	3.2
66	4.2	0.0	4.1	0.0	3.8	0.0	4.2	5.0	0.0	0.0	4.0	0.0	3.0	—
B	0.0	0.0	0.0	0.0	0.0	0.0	1.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
F	5.0	0.0	5.0	0.0	4.2	0.0	4.8	4.0	1.0	1.8	4.8	0.0	—	2.0
P	3.0	0.0	3.0	0.0	1.0	0.0	3.5	4.0	0.0	0.0	2.5	0.0	0.0	—
T	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.5	0.0	0.0	0.0	0.0	0.0	0.0
None	4.5	1.5	4.5	1.8	4.8	1.2	5.0	5.0	2.0	3.0	4.2	1.0	3.5	3.8

[a] Figures indicate degree of severity of symptoms in ascending order from 1 to 5, inclusive. Zero indicates no observed increase in severity of symptoms following a second inoculation.

shows severe injury produced following introduction of the virulent strain 1 into a healthy plant and into one recovered from the less virulent strain 2.

No evidence of injury was noted when less virulent strains were introduced into plants recovered from more virulent strains. Since no satisfactory method is known of separating mild strains of the virus from mixtures with more virulent strains, no attempt was made to determine whether the mild strains were superimposed on the more virulent strains in such tests. However, by taking advantage of the fact that the virulent strain 1 will infect the sugar-beet variety S. L. 68 and the virulent strain 3 usually does not, it was demonstrated that strain 1 may be readily superimposed on strain 3 in water pimpernel even though it does not cause added symptoms.

It may be noted that plants recovered from the virulent strains 3, 6, and B suffered a certain amount of injury following inoculation with the very virulent strain 22C. The injury, however, was not comparable in severity to that produced by 22C alone. Under favorable conditions for growth the plants showed added symptoms following the introduction of the second strain, recovered a second time, and produced plants that were more or less normal except for reduced vigor.

In general these results indicate that plants that have recovered from 1 curly-top virus strain may be injured by the introduction of any strain of the virus more virulent than the one from which the plant recovered. Plants carrying strains of low virulence are injured as severely as nonrecovered plants; plants carrying strains of medium severity are injured somewhat less and appear to be protected to some degree against severe types of injury produced by very virulent strains alone.

DISCUSSION.—The factors involved in the failure of strains of curly-top virus to offer an appreciable amount of protection against infection by other strains and against injury by strains more virulent than the one present in the infected plant, are still far from clear.

The theory most often advanced to account for cross protection between plant-virus strains postulates that there is such a complete utilization by the first virus of the materials essential for virus increase that the sec-

FIG. 2. Plants of water-pimpernel infected with curly-top virus. A) Previously healthy plant inoculated with virus strain 1. B) Plant recovered from strain 2 and reinoculated with the same strain. C) Plant recovered from strain 2 and reinoculated with strain 1. Strain 1 has a high degree of virulence and strain 2 a low degree of virulence. All plants were about the same size when plants A and C were inoculated with strain 1 and plant B was reinoculated with strain 2.

ond virus, if related, cannot find essential materials on which to increase. Bawden and Kassanis (1) proposed a second theory in which it is suggested that there may be a limited number of sites for virus increase within the plant. When all of the available sites are occupied by the first virus the plant becomes immune to infection by other viruses having the same site requirements for increase.

Obviously, in the case of the curly-top virus, neither all of the materials nor all the sites essential for virus increase are utilized by the first virus that invades the plant. Actually, with respect to ability to increase, the second virus, at least in the early stages of its invasion of the plant, seems to have an advantage over the first. This must be true, for evidence indicates, in some cases at least, that the challenging virus eventually reaches concentrations approximating those of the first virus introduced.

It would seem that this condition could arise only if the first virus reached a stage in which its increase were retarded by factors operating within the plant. It seems probable that such conditions may exist in association with the high adsorptive properties of the curly-top virus.

The curly-top virus has an affinity for adsorption on some types of proteins. For instance, the virus is carried out of suspension in plant juices by precipitates formed as a result of heating, of the addition of alcohol, and of standing at room temperature and by precipitates formed naturally in fresh juice of water pimpernel. Because of these high adsorptive properties, most of the virus in a diseased plant may exist in an adsorbed or aggregated state in which it is not free to increase and utilize fully all of the essential elements or sites that are available. Therefore, when a second virus strain is introduced, particularly by the natural vector which may have the power to disassociate virus aggregates, it encounters an abundant supply of available elements or sites for increase and is free to multiply at a relatively rapid rate until the phenomenon of adsorption begins to affect adversely its reproductive capacity.

Phenomena concerned with adsorption or aggregation of the curly-top virus may be involved in recovery. Undoubtedly, one of the factors involved in the production of severe symptoms by virulent strains is virus concentration. As concentrations rise to a peak following initial introduction of virus into the plant, the plants are injured severely; as concentrations drop, the plants tend to recover. Recovery, therefore, may be due to adsorptive or aggregative affinities that operate to reduce the level of concentration of active virus below that capable of causing severe symptoms.

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THE RELATIONSHIP OF ROOT-KNOT NEMATODES TO GRANVILLE WILT RESISTANCE IN TOBACCO [1]

G. B. Lucas, J. N. Sasser, and A. Kelman

SUMMARY

The relationship between infection by root-knot nematodes and severity of Granville wilt in a moderately wilt-resistant tobacco variety, Dixie Bright 101, was investigated in greenhouse experiments. Tobacco plants were grown in soil to which *Pseudomonas solanacearum* and *Meloidogyne incognita acrita* were added alone and in combination. Plants grown in soil infested with both pathogens exhibited wilt symptoms earlier and to a greater extent than plants grown in soil infested only with the wilt bacterium. The role of the nematodes appeared to be principally that of providing wounds through which the bacteria may enter. The development of wilt symptoms in plants growing in soil infested with both pathogens was comparable to that in plants inoculated by pouring a bacterial suspension on freshly cut roots.

When bacteria were placed in contact with roots 24 or 48 hours after wounding, disease indices were lower than when the bacteria were added immediately after wounding. This pointed to the rapid healing ability of the root tissues and the fact that bacteria enter mainly through fresh wounds.

Plants inoculated with a concentrated suspension of bacteria developed wilt symptoms much more rapidly than those inoculated with 1/10th as many bacteria, and the symptoms were much more severe. In the presence of nematodes, however, severity of wilt was greatly increased at both levels of bacterial inoculum.

These experiments indicate the advisability of controlling root knot in fields heavily infested with the Granville wilt bacterium in order to gain maximum benefit from the use of wilt-resistant varieties.

A relationship between the invasion of roots by root-knot nematodes (*Meloidogyne* spp.) and the severity of southern bacterial wilt or Granville wilt caused by *Pseudomonas solanacearum* E.F.Sm. often has been postulated but never conclusively demonstrated. This relationship was suggested in part by field observations and in part by evidence that the wilt bacterium is essentially a wound invader (6). Experimental evidence in support of this concept was presented by Hunger (4), who demonstrated in a limited test that tomato plants growing in soil infested with root-knot nematodes were readily infected by the wilt bacterium, whereas plants in nematode-free soil did not contract the wilt disease.

Following the development and extensive planting of wilt-resistant varieties such as Oxford 26 and Dixie Bright 101 in North Carolina, these varieties appeared to be less resistant to Granville wilt in fields infested by root-knot nematodes than in nematode-free fields. To obtain more precise information on the suggested disease interactions, greenhouse investigations were initiated. The objectives were to determine 1) whether the severity of Granville wilt in a resistant variety is greater in the presence of root-knot nematodes than in their absence, 2) whether the apparent decrease in resistance to wilt can be attributed simply to nematode wounding, and 3) whether inoculum level influences disease severity in both the presence and absence of the nematode. A preliminary report of these studies has been made (8).

MATERIALS AND METHODS.—Seedlings of Dixie Bright 101, a tobacco variety moderately resistant to Granville wilt, were grown in fine sandy loam soil that had been fumigated with methyl bromide (bromomethane) gas prior to use. Plants were grown to transplant size in 2-in. pots and were then repotted into 6-in. pots when they were approximately 5–7 cm in height. In order to minimize root injury at repotting, an attempt was made to keep intact the entire ball of soil containing the roots. Groups of 20 uniformly sized plants each received 1 of the following 6 separate treatments: 1) approximately 5 g of tomato or tobacco roots containing egg masses of the cotton root-knot nematode, *M. incognita acrita* Chitwood added to the soil in each pot; 2) 50 ml of a suspension of *P. solanacearum*, containing approximately 20×10^6 bacteria per ml from a 3-day-old potato-dextrose agar culture, poured around the roots of each plant; 3) 50 ml of a bacterial suspension, containing approximately 20×10^7 bacteria per ml, poured around the roots of each plant; 4) nematodes plus dilute bacterial suspension as in 2; 5) nematodes plus concentrated bacterial suspension as in 3; and 6) controls, receiving neither pathogen. The bacterial suspensions were poured on the soil around the seedlings 24 hours after transplanting.

The tobacco or tomato plants that provided the root-knot nematode inoculum were grown in the greenhouse (21–32°C) in 6-in. pots in soil infested with *M. incognita acrita* for approximately 90 days to permit production of numerous egg masses. The roots were washed free of soil, then chopped, mixed, and separated

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into 5-g portions. The bacterial inoculum was prepared from cultures of the virulent colony type of *P. solanacearum*, which had been checked for pathogenicity by the tetrazolium-agar technique (7). The above experiments were performed 3 times. Two preliminary tests with minor variations were also completed.

To determine the effect of root injury on Granville wilt incidence, roots in the ball of soil on 1 side of each of 60 transplants were cut with a sharp knife. The plants were then divided into 3 groups of 20 plants each, and 50 ml of the concentrated bacterial suspension was poured over the injured roots of each plant at one of the following intervals after transplanting: 1) immediately, 2) 24 hours, and 3) 48 hours. For comparison, plants with roots not injured by cutting were inoculated with the bacterial suspension immediately and 24 hours after adding nematode inoculum to soil.

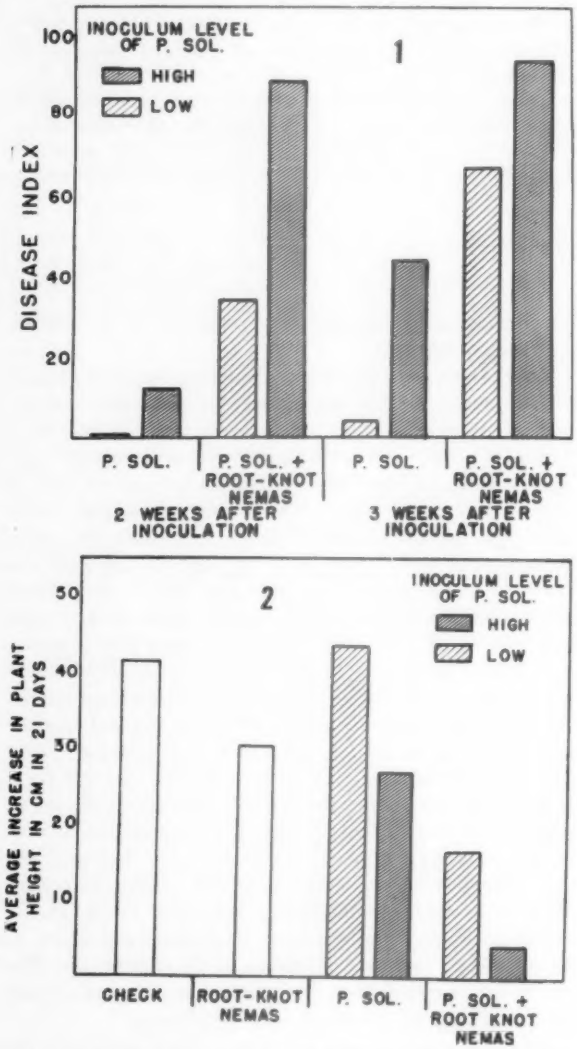

FIG. 1–2. Fig. 1. Granville wilt disease indices in Dixie Bright 101 tobacco plants 2 and 3 weeks after inoculation with *Pseudomonas solanacearum* and *Meloidogyne incognita acrita* alone and in combination.—Fig. 2. Average increase in height of Dixie Bright 101 tobacco plants following inoculation with *P. solanacearum* and *M. incognita acrita* alone and in combination.

Greenhouse temperatures ranged from 21–27°C at night to 27–38°C in the daytime. Height measurements and wilt readings were taken at weekly intervals. Disease indices were prepared following the method used by Winstead and Kelman (10).

EXPERIMENTAL RESULTS.—Within 1 week after transplanting and inoculating, symptoms of Granville wilt were more advanced in plants growing in soil infested with both pathogens than in plants growing in soil infested only with bacteria. In a representative test (Fig. 1) this difference was pronounced at the end of 2 weeks, and at the end of 3 weeks most of the plants infected with both pathogens were dead (Fig. 1 and 3). The concentrated bacterial suspension induced more severe wilt symptoms than the less concentrated suspension. In the presence of nematodes, however, severity of wilt was greatly increased at both inoculum levels.

Data on height measurements indicated an inverse correlation between the increase in plant height following inoculation and the severity of disease (Fig. 2). At the end of 21 days the growth of plants inoculated with the dilute bacterial suspension was comparable to that of control plants. Plants inoculated either with root-knot nematodes alone or the dense bacterial suspension alone were only moderately stunted, whereas those plants inoculated with both pathogens were severely stunted.

In preliminary tests it was found that moderate to severe wilt developed in plants in nematode-free soil if bacterial inoculum was added immediately after transplanting. If bacteria were added 24 hours later, disease severity was markedly reduced. These observations indicated 1) that roots wounded in transplanting were serving as infection courts for the bacteria and 2) that delay in adding inoculum might permit some wound healing to occur. A test was completed, therefore, to compare mechanical wounding with the effects of the nematode in relation to disease severity and also to confirm observations that delay in adding bacterial inoculum resulted in decreased wilt. Results are summarized in Table 1. The most severe wilt symptoms appeared in 2 groups of plants: 1) those wounded with a knife and inoculated immediately with bacteria (Fig. 4, left), and 2) those not wounded mechanically but inoculated with nematodes and bacteria at the time of transplanting. Adding the bacteria 24 hours later in both cases produced a considerable reduction in the numbers of diseased plants (Fig. 4, right). This reduction due to delay in adding bacteria, however, was less evident in plants growing in the nematode-infested soil. A further decrease in severity of wilt occurred when the bacteria were added 48 hours after wounding.

DISCUSSION AND CONCLUSIONS. — The experiments herein reported indicate that the root-knot nematodes greatly facilitate infection of Dixie Bright 101 by the

FIG. 3. Dixie Bright 101 tobacco plants 21 days after inoculation with *P. solanacearum* and *M. incognita acrita*. Left: Root-knot nematodes added at transplanting and *P. solanacearum* (concentrated suspension) added 24 hours after transplanting. Right: *P. solanacearum* alone (concentrated suspension) added to soil 24 hours after transplanting.

FIG. 4. Dixie Bright 101 tobacco plants 11 days after transplanting. Left: Roots wounded by cutting with a knife and *P. solanacearum* (concentrated suspension) added immediately after wounding. Right: Roots wounded by cutting with a knife and *P. solanacearum* (concentrated suspension) added 24 hours later.

Granville wilt bacterium. Although the penetration of roots by root-knot nematodes does not usually result in visible necrosis of the tissue, a certain amount of injury to cells must occur. In studies on the entrance of larvae into roots, Christie (2), showed that the larvae usually push the cells apart and pass between them; however, at times the parasite passes through cells. In any case certain cells are crushed and destroyed. After penetration of the root, the larvae advance through the cortical tissues and, by the fourth day, orient themselves with their head ends buried in the region of the clearly differentiated pericycle of the stele and endodermis of the cortex (3). In this manner the nematodes may provide the initial openings in the root and the pathways through the cortex for the wilt bacterium.

In greenhouse investigations, Dixie Bright 101 tobacco has been found to be highly susceptible to the root-knot nematode species known to occur in the eastern United States and moderately resistant to Granville wilt when root-inoculations were used in the nematode-free soil (5). In the development of inoculation techniques for evaluation of resistance to Granville wilt, it was found that expression of wilt resistance could be modified by variation in the age of plants, inoculum level, and type of inoculation procedure (10). The data obtained in these tests with both pathogens indicated that mass invasion of the roots by larvae in the presence of a relatively high population of bacteria would bring about severe wilt in a moderately resistant variety. There was no indication from these tests that increased susceptibility was associated with action by the nematode on the host other than by providing multiple infection courts for the bacterium.

TABLE 1.—*Relative severity of Granville wilt in Dixie Bright 101 tobacco plants inoculated with Pseudomonas Solanacearum alone, with and without wounding, and in combination with Meloidogyne incognita acrita*

Type of injury	Time from injury to adding of bacteria	Disease index[a] at indicated interval after transplanting	
		6 days	13 days
	Hours		
Wounded by cutting	0	48	100
	24	2	55
	48	0	29
Not wounded	0	7	67
Nematode infection	0	15	90
	24	3	77
LSD at 0.05 level		9.3	15.9
LSD at 0.01 level		12.4	21.1

[a] Each reading based on 20 plants.

Examination of roots of plants growing in soil infested with both pathogens revealed that relatively few nematodes produced galls in the roots of plants affected by the wilt disease. The necrosis of tissues that characterize infection by the wilt bacterium presumably prevented the establishment of the host-parasite relationship essential for maturation of the root-knot nematode.

Rapid healing of injured roots is a suggested explanation for the decrease in incidence of wilt when the bacteria were added 24 or 48 hours after wounding roots directly. Evidence for rapid healing within the time limits indicated has been presented by Artschwager and Starrett (1). These investigators have shown that, under conditions of high humidity and temperature, plant tissues may suberize within 24 hours after wounding and form periderm within 48 hours. A 24-hour delay in adding bacteria to plants placed in nematode-infested soil did not result in as great a reduction of wilt incidence as a similar delay for plants with cut roots. Presumably this was due to the fact that nematode larvae invaded roots over a period of several days and fresh wounds were being made during this period.

In most tests, wilt symptoms developed in plants inoculated with bacteria but not wounded directly. This was particularly true when high concentrations of bacterial inoculum was used. The incidence of wilt in these plants can be attributed to infection through breaks in the roots that occurred in transplanting. Even though the entire ball of earth was carefully removed from thumb-pots in transplanting, a certain number of rootlets were inevitably broken.

The interaction between root-knot nematodes and

Granville wilt differs from the relationship between these nematodes and black shank of tobacco caused by *Phytophthora parasitica* Dast. var. *nicotianae* (B. de Haan) Tucker, wherein factors in addition to wounding appear to be involved (9). This is explained in part by the evidence that *P. solanacearum* is essentially a wound invader, whereas *P. parasitica* var. *nicotianae* is capable of penetrating uninjured tissue directly.

The important conclusions derived from these tests concern the increased damage that may occur to tobacco from the interaction of the 2 pathogens under field conditions. It is evident that in the absence of nematode-resistant varieties, it will be advisable to control root knot, either by soil fumigation or crop rotation, in fields where Granville wilt is present. Otherwise full value will not be received from the use of wilt-resistant varieties such as Dixie Bright 101.

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SEASONAL FLUCTUATIONS AND HOST SPECIFICITY OF ROOT-KNOT NEMATODE POPULATIONS IN TWO-YEAR TOBACCO ROTATION PLOTS[1]

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SUMMARY

A study was made of the seasonal fluctuations and host specificity of root-knot nematode populations in 2-year tobacco rotation plots. The following observations and interpretations are given: 1) root-knot nematode populations fluctuate widely from time to time throughout the year; 2) such studies may be of considerable value in interpreting the results of crop rotation experiments designed to evaluate various cropping systems; 3) all of the rotations, while not eliminating the nematodes, served to keep them in check, thus making it possible to grow a good crop of tobacco; 4) in the continuous tobacco plots, the population of root-knot nematodes consisted of 2 species, *Meloidogyne incognita* and *M. arenaria*, that apparently had become stabilized in their ability to infect and reproduce on tobacco, but they did not infect cotton; and 5) a population of *M. incognita acrita* in 1 of the cotton-tobacco rotation plots severely attacked cotton but did not attack tobacco. In other plots of the cotton-tobacco rotation, the population of *M. incognita acrita* attacked both tobacco and cotton, indicating the existence in this form of strains or races that differ in ability to attack these crops. The laboratory technique employing the indicator plant method appears to be reliable for measuring soil populations of root-knot nematodes.

Crop rotation has long been recognized as an effective and practical means of controlling nematode diseases of various crop plants (1, 4, 6, 11, 13, 14). In principle, plant pathogenic nematodes, being obligate parasites, depend upon living roots of suitable host plants for their development and reproduction. The planting of susceptible crops on the same land year after year permits parasitic nematodes to build up to high population levels in the soil, thus making it unfit for the profitable production of these crops. Use of resistant crops, in rotation with susceptible crops, interrupts the development of these parasites and reduces losses caused by them. Application of

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this principle to control root knot, however, is complicated by many factors among which are 1) the wide host range of the root-knot nematodes, and 2) the existence of species and of strains within species that exhibit different host preferences.

Studies of the influence of certain alternate crops on the control of root knot and on the yield and value of tobacco in North Carolina started with the establishment of a rotation experiment at the McCullers Experiment Station near Raleigh, North Carolina, in 1937. The experiment was located on land known to be severely infested with root-knot nematodes. Various cropping sequences were used in 2-, 3-, and 4-year rotations. Data were taken each year from 1937 to 1952 on yield, grade distribution and value of tobacco, and on root-knot incidence after harvest. Measurements of these gross effects have been valuable in plotting the course of nematode diseases on tobacco and also of crop performance year after year. The data thus obtained, while indicating that marked fluctuations in nematode populations may have taken place, do not give an adequate picture of the magnitude of these fluctuations or when they occurred. It seemed desirable, therefore, to make a census of nematode populations in certain selected rotation plots at intervals that might indicate 1) the numbers surviving the winter, 2) the numbers surviving land preparation in the spring, and 3) the magnitude of increase or decrease in numbers during the growing season. The objectives were 1) to gain a statistical appraisal of the sampling and experimental techniques employed, 2) to study the population changes of root-knot nematodes in six 2-year rotations throughout a 1-year cycle for both tobacco and the alternate crops, and 3) to evaluate population studies in interpreting the results of rotation experiments.

These investigations were made in 2 separate phases. The first phase, dealing with seasonal population changes, was conducted in 1949 and 1950 and was terminated by the absence of the senior author. Upon his return, the second phase, concerning primarily the identity of root-knot nematode species and host-parasite relationships, was undertaken in 1953.

All root-knot nematodes were classified under the species *Heterodera marioni* (Cornu 1879) Goodey, 1932, until Chitwood (2) reassigned them in 1949 to the genus *Meloidogyne*, based upon detailed morphological studies. He described 5 species and 1 subspecies and later (3) identified another subspecies. In 1953, Loos (7) described a new species from Ceylon.

In the interval between 1949 and 1953, much was learned concerning the morphology and host-parasite relationships of the various root-knot nematode species (2, 10). In the light of this new knowledge, it seemed likely that a study of the root-knot nematode populations of certain rotation plots at the McCullers station would facilitate the interpretation of data obtained in the first phase of the investigation. Although the rotation experiment was terminated in 1952, it was still possible to locate the original plots. Therefore, samples were collected in the spring of 1953 for species determinations and greenhouse inoculation tests. Special attention was given to the host-parasite relationships of these populations in tobacco, cotton, and peanuts. The results of both phases of the work are herein presented. An abstract of the first phase has been published (9).

MATERIALS AND METHODS. — Root-knot nematode population studies were made on 2-year rotation plots of cotton-tobacco, corn-tobacco, peanuts-tobacco, weeds-tobacco, and (oats-weeds)-tobacco. The original design of the experiment was such that it permitted nematode population studies on soil from tobacco plots and the alternate crop plots the same year. Alternate crops grew on the plots in series I in 1948, while tobacco grew on the plots in series II. In 1949, tobacco grew on plots in series I and alternate crops in series II. Each series contained 2 replications of the rotations being studied. Two plots of continuous tobacco were included in each series to serve as checks. Plots were 1/20 acre in size.

Nematode population determinations were made from soil collected February 23, May 23, and November 23, 1949; and March 22, 1950. A soil-sampling tube 1 in. in diameter was used to collect the soil. Thirty-five borings from the top 8 in. of soil were taken at random from each plot and composited. These composited samples were then taken to the greenhouse and thoroughly mixed by running through a riffle. Three 4-in. pots were filled with this mixture. A tobacco plant (402 variety), grown in nematode-free soil, was transplanted to each pot to serve as an indicator. The plants grew for 30 days in the greenhouse with temperatures ranging from 70 to 80°F. At the end of this period, the roots were carefully separated from the soil by washing with a gentle stream of water. In the laboratory the roots were cut into pieces approximately 1 in. long and weighed. A 2-g sub-sample was floated on water in a transparent dish with light transmitted from underneath. The root-knot galls were then counted and the total number of galls per indicator plant was calculated.

Godfrey (5), who first used the indicator-crop method to study the degree of soil infestation with the root-knot nematode, found a high correlation between gall count on roots of tomato and actual numbers of contained nematodes. Since one of the purposes of this investigation was to get a reliable estimate of viable eggs and larvae in a given amount of soil at different seasons of the year, counting the number of galls on the roots of the indicator plant seemed to be the logical approach. For the second phase of the work, namely, a determination of the species present in the various rotation plots and further studies on host-parasite relationships, soil was collected from each of the 2-year rotation plots in a manner already described. These samples were taken in the spring of 1953. Enough soil was collected from each plot to fill nine 4-in. pots. A tobacco seed-

ling (Dixie Bright 101 variety), grown in nematode-free soil, was planted in each of 3 pots as an indicator for root knot. Cotton (Coker 100 wilt resistant) was planted in 3 pots and peanuts (Spanish variety) were planted in 3 pots. The tobacco was allowed to grow for 30 days, and a root-knot gall count was made at that time. The cotton and peanuts were allowed to grow for 3 months and rated for root knot as described by Smith and Taylor (12).

To determine whether or not the nematodes that had infected either tobacco or cotton indicator plants were cross infective, reciprocal infestations were made of steamed soil, and the plants were allowed to grow for 60 days in the greenhouse at temperatures ranging from 70–80°F. The plants were then examined and rated for root-knot infection.

EXPERIMENTAL RESULTS.—Seasonal fluctuations in root-knot nematode populations in 2-year rotation plots are shown graphically in Figure 1. A separate graph is used for each rotation. In each case, data from series I and series II plots are plotted separately in order to facilitate interpretation of the data. In these graphs, which cover a 1-year cycle, a shift in the type of line, from solid to broken, or *vice versa*, indicates a change in crops.

In the continuous tobacco plots, the nematode population levels at each sampling date were similar in both series. During the growing season of 1949, the population rose sharply as shown by the high numbers obtained in November. In the corn-tobacco rotation, the infestation developed to high levels during the 1949 season in both the corn and tobacco plots. In the peanut-tobacco rotation there was no appreciable increase on peanuts during the 1949 growing season. Tobacco, however, built up a moderately high population level by November. The data from cotton-tobacco rotation plots revealed nematode population trends entirely different from those noted in the tobacco-peanuts rotation. During the 1949 growing season the nematodes apparently developed much more abundantly on cotton than on tobacco, as shown by the November samples. In the weeds-tobacco rotation, degree of infestation rose sharply on tobacco and remained high on weeds during the 1949 growing season. In the (oats-weeds)-tobacco rotation, the population rose sharply during the growing season on tobacco but failed to build up appreciably in the weeds plots.

There was considerable variation in the root-knot nematode gall counts on indicator plants among plots and among indicator plants of the same plot, as well as among dates on which the sample was taken. In order to assess the efficiency of the laboratory technique of measuring soil populations in terms of a coefficient of variation, it was necessary to attempt to stabilize the within-plot variance by using a logarithmic transformation, $y = \log (x + 1)$ where x is the observed count. In terms of logarithms, a coefficient of variation of 16 per cent was calculated.

TABLE 1.—*Root-knot nematode gall counts from indicator plants (variety Dixie Bright 101 tobacco) and root-knot index of cotton and peanuts grown in soil collected from 2-year tobacco rotation plots February 1953*[a].

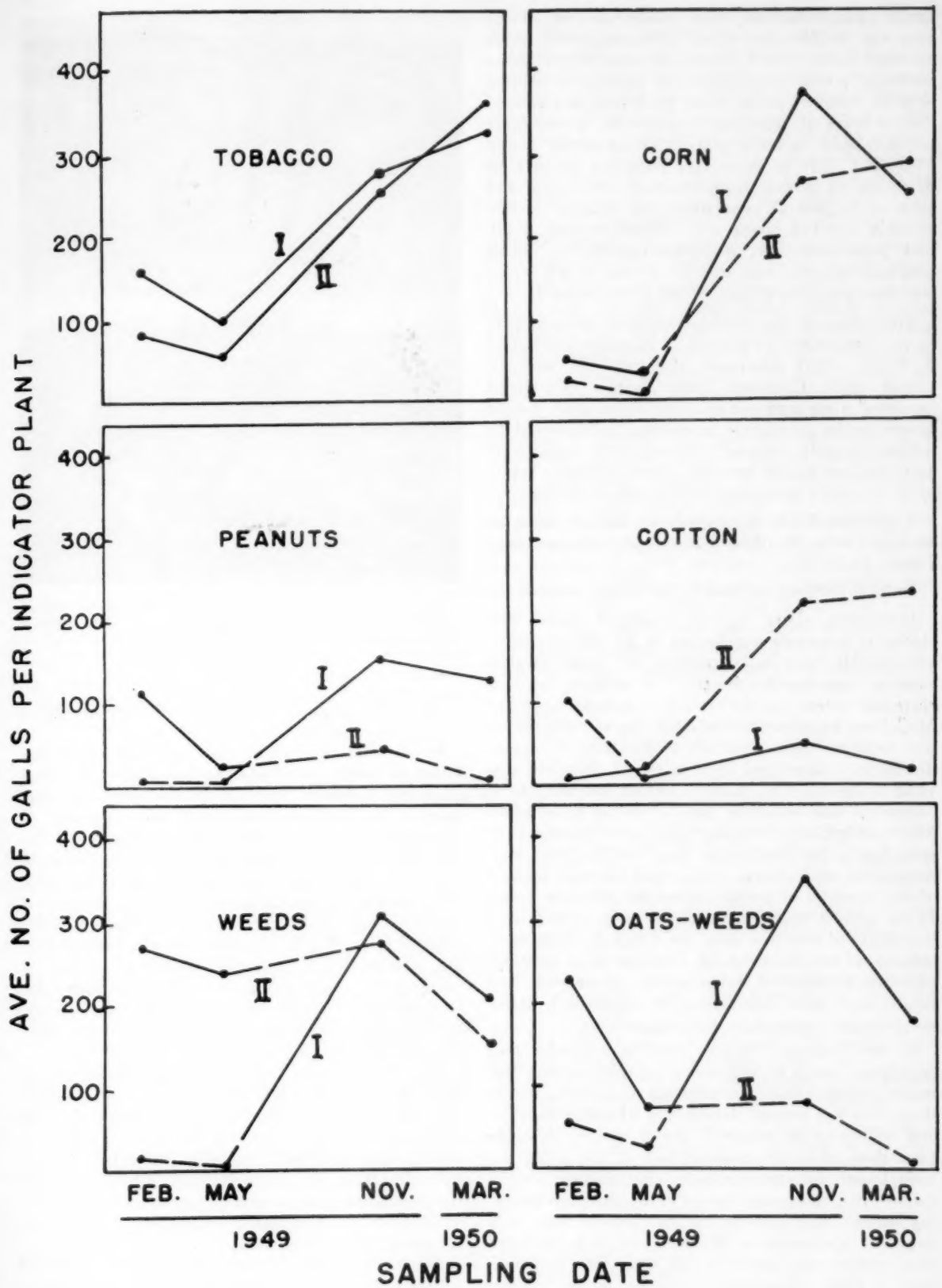
Rotation	1952 Crop	Plot	Average no. galls per tobacco plant	Average root knot index[b] (cotton)
Series I				
Corn-tobacco	Corn	1-A	85	1.5
		1-B	12	3.0
Peanuts-tobacco	Peanuts	2-A	463	0
		2-B	23	1
Oats-weeds-tobacco	Oats-weeds	3-A	23	0
		3-B	1	4
Weeds-tobacco	Weeds	4-A	20	0
		4-B	59	2
Cotton-tobacco	Cotton	5-A	11	2.5
		5-B	0	4.0
Tobacco-tobacco	Tobacco	6-A	1136	0
		6-B	853	0
		6-C	1285	0
		6-D	856	0
Series II				
Corn-tobacco	Tobacco	7-A	323	2
		7-B	269	4
Peanuts-tobacco	Tobacco	8-A	384	1
		8-B	1667	0
Oats-weeds-tobacco	Tobacco	9-A	640	1
		9-B	963	0
Weeds-tobacco	Tobacco	10-A	1345	0
		10-B	373	1
Cotton-tobacco	Tobacco	11-A	100	3.5
		11-B	421	2.0
Tobacco-tobacco	Tobacco	12-A	361	0
		12-B	435	0
		12-C	623	0
		12-D	104	0

[a] None of the populations produced symptoms on peanuts (Spanish variety).

[b] Relative root-knot index based on: 0 = no infection, 1 = trace of infection, 2 = light infection, 3 = moderate infection, and 4 = heavy infection.

The reaction of tobacco, cotton, and peanuts to the root-knot nematode populations in the soil collected from the various plots in the spring of 1953 are given in Table 1. Tobacco was attacked by all of the populations except the one in plot 5-B of series I, a

FIG. 1. Seasonal fluctuations in root-knot nematode populations in 2-year tobacco rotation plot soils. The solid lines indicate populations after tobacco; the broken lines, after alternate crops. Roman numerals I and II represent the 2 series of plots.


AVE. NO. OF GALLS PER INDICATOR PLANT
400
300
200
100
TOBACCO
I
II
CORN
I
II
PEANUTS
I
II
COTTON
II
I
WEEDS
II
I
OATS-WEEDS
I
II
FEB. MAY NOV. MAR.
1949 1950
FEB. MAY NOV. MAR.
1949 1950
SAMPLING DATE

cotton-tobacco rotation. The species present in this plot was *M. incognita acrita* Chitwood, 1949, which attacked cotton severely (root-knot index 4.0). Tobacco seedlings grown in steamed soil infested by adding severely infected cotton roots from this plot showed only a trace of infection. Populations in the other cotton-tobacco rotation plots (5-A of series I and 11-A and 11-B of series II) consisted entirely of *M. incognita acrita*, which attacked both cotton and tobacco. In general, populations that attacked tobacco severely attacked cotton only slightly or not at all, and populations that multiplied rapidly on cotton attacked tobacco only slightly or not at all. This was true for plots of both series I and series II.

Populations in the continuous tobacco plots of both series I and series II consisted of *M. incognita* (Kofoid & White, 1919) Chitwood, 1949, and *M. arenaria* (Neal, 1889) Chitwood, 1949. Neither reproduced on cotton when grown in soil from those plots. Cotton grown in the greenhouse in steamed soil infected by adding severely infected tobacco roots from these plots was not visibly attacked. Some immature stages of *M. incognita* were found when roots were dissected.

Population levels were generally higher following tobacco (series II) than following the alternate crop (series I).

Peanuts were not infected by any of the populations.

Discussion.—Data herein presented show that studies of nematode populations in the soil may be of considerable value in interpreting the results of crop rotation experiments designed to evaluate various cropping systems on the control of root knot. In the McCullers experiment conducted since 1937, all of the rotations have reduced losses from nematode disease in tobacco and have increased the yield and value of the crop (1). Little is known, however, of the history of the nematode populations in these plots. The examination of tobacco roots after harvest each year has given worthwhile data on the total root damage for each season, but nothing has been learned of the activities of nematodes on the alternate crops. In the present study emphasis has been shifted from the effects of rotations upon the nematode diseases of tobacco to the effects of all crops in these rotations upon the activities of the nematodes themselves. The results have given information on nematode behavior not hitherto obtained in this experiment.

In the continuous tobacco plots, it is notable that population trends in both series are comparable. This might indicate that the nematode population within these plots has become stabilized in its ability to infect and reproduce on tobacco. The 2 species identified from these plots, *M. arenaria* and *M. incognita*, are both known to attack tobacco, but neither attacks cotton (10). There are reports of *M. incognita* attacking cotton (8); however, in the present tests only immature specimens of *M. incognita* were dissected from cotton roots grown in soil from the continuous tobacco plots. Apparently, variation in the populations of *M. incognita*, along with different varieties of cotton used, account for this difference.

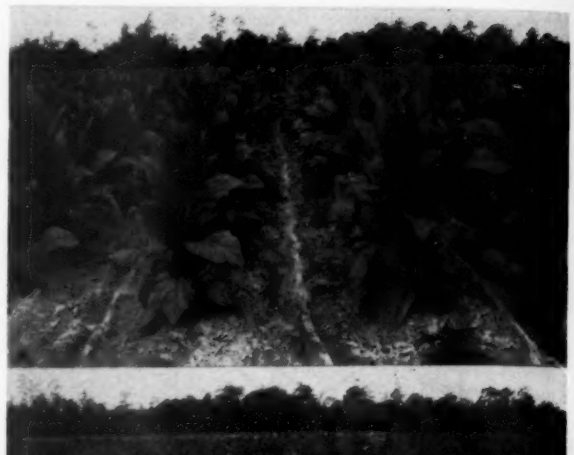


Fig. 2. Two plots of tobacco in the McCullers rotation experiment photographed in July 1949. A) After peanuts. B) Continuous tobacco.

In the peanut rotation, the root-knot population failed to build up on peanuts but developed moderately on tobacco. This would be expected since peanuts are not attacked by *M. incognita* and *M. incognita acrita*, the forms present in these plots. The resistant crop in this rotation, while failing to eliminate the nematodes, serves to keep them in check, thus making it possible to grow a good crop of tobacco (Fig. 2, A) as compared to the continuous tobacco plots (Fig. 2, B). The fact that peanuts were not infected by *M. arenaria*, which was present in some of the continuous tobacco plots, indicates that this population of *M. arenaria* is different from the *M. arenaria* in Georgia (2) and other states where peanuts are severely attacked by this species.

The cotton rotation presents a population picture quite the opposite of that in the peanut rotation. Here, the data indicate the presence of a population of the root-knot nematode in series I plot No. 5-B (Table 1) that develops abundantly on cotton but not on tobacco. In series II of the cotton-tobacco rotation, however, the population attacks both cotton and tobacco (plots 11-A and 11-B), although cotton appears to be a more favorable host than tobacco for reproduction, as shown by the rapid increase in population following cotton (Fig. 1). The moderately high population of

nematodes following cotton (series I, Fig. 1) and the decrease in population following tobacco indicates a strain that multiplies abundantly on cotton but very weakly on tobacco. Further evidence of this is shown in Table 1, series I, in which the population that severely attacked cotton was able to attack tobacco only slightly (plots 5-A and 5-B). The results of the host-parasite relationship studies of these 2 nematode populations conducted in the second phase of the investigation has helped explain the somewhat unusual population changes in the cotton-tobacco rotation observed in the first phase of the work (Fig. 1). The root-knot nematode population in these plots consisted of the subspecies *M. incognita acrita*, which is known to vary in ability to attack certain hosts (8). Apparently, physiological strains or races exist within the subspecies.

Root-knot nematodes in the corn rotation plots developed abundantly in both corn and tobacco, reaching high population levels in the fall of 1949. Clayton et al (4) have shown that corn, while rarely suffering root-knot damage, does not actually reduce root-knot nematode populations. Annual root-knot indices on tobacco in corn rotations at McCullers[2] showed a gradual reduction between 1937 and 1946, indicating that root-knot nematode populations developed less and less each season. Since 1947, however, the root-knot indices have been steadily increasing. This might indicate that corn was a noncongenial host to the original population of root-knot nematodes and that later 1 or more strains appeared that thrive on corn as well as on tobacco. Had soil population studies been started in these plots a few years earlier, it might have been possible to detect changes in nematode-host relations.

The population build-up on tobacco in series I in both the weeds and oats-weeds rotations show comparable trends, but the series II data are strikingly different. In series II of the weeds rotation, the population was high in the spring of 1949 and remained high. In series II of the oats-weeds rotation, however, there was a sharp drop from February to May, 1949, and the population remained low thereafter. A possible explanation for these different population trends may be associated with the dissimilarity of cultural practices employed. In the weeds plots, the soil was undisturbed throughout the winter, spring, and summer. Weeds made an early start in the spring, and during the growing season a heavy cover of horse-weed (*Erigeron canadensis* L.), ragweed (*Ambrosia artemisiifolia* L.), and buttonweed (*Diodia teres* Walt.) developed. Observations showed that roots of 2 of these weeds, horse-weed and buttonweed, were infected with root-knot nematodes. It is likely that root-knot began to develop early in these weeds and that fragments of infected roots were taken in the May, 1949, samples. In oats-weeds plots of series II, oats were sown in the fall of 1948 and served to shade out weed growth until after the grain was harvested in early summer. Oats, a crop highly resistant to root knot (10), did not maintain a high population throughout the spring. Moreover, the weed stand, which came in late June, failed to build up a population of nematodes comparable to that on the early established weeds in the weeds rotation.

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[2] Unpublished data.

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HISTOLOGICAL STUDY OF PATHOGEN-SUSCEPT RELATIONSHIPS BETWEEN PHYTOPHTHORA INFESTANS AND DERIVATIVES OF SOLANUM DEMISSUM [1]

Virginia Rogers Ferris

SUMMARY

The interactions between leaf tissue of derivatives of *Solanum demissum* and several races of *Phytophthora infestans* were investigated histologically. Techniques for inoculation and incubation were standardized, and all plants were maintained at 18°C following inoculation. The histological examinations were made on cleared whole leaves and on sectioned material. In susceptible plants, mycelium was found in tissue distant from the site of penetration within 17–28 hours following inoculation. In resistant plants, short hyphae often were seen between mesophyll cells near the site of penetration, but widely ramifying mycelium was never observed. A necrotic response was evident in the resistant plants at most only a few hours before such a response developed in susceptible plants. Differences were noted in the rapidity with which the necrotic response developed in the different varieties. Plants duplex for a given resistance gene were inoculated with races pathogenic to plants simplex for the same gene. After 4 days the duplex plants had the macroscopic appearance of resistant plants. Histological examinations showed that after 42–49 hours many of the lesions on these plants were different in appearance from the lesions of the simplex resistant or of the susceptible plants. A susceptible reaction was obtained in the duplex plant when the inoculum consisted of a culture reisolated after serial passages through plants of the same genotype.

It is known that resistant leaflets of *Solanum tuberosum* L. are penetrated by *Phytophthora infestans* (Mont.) d By., but comparatively little detailed study has been made of the interactions between the resistant potato leaf tissue and the fungus.

The first description of penetration and of the establishment of mycelium in susceptible leaves was by de Bary (7, 8). This description was followed later by others (11, 21, 26). Crosier reported in 1933 that the fungus penetrated leaves of resistant plants of *Solanum demissum* Lindl. (6). Hori (9) in 1935 reported that when plants of about 80 species, which belonged to several families, were inoculated with swarmspores of *P. infestans*, penetration occurred in nearly all. Penetration of resistant and of susceptible varieties of the solanaceous plants tested did not appear to differ. In resistant varieties the invaded cells became necrotic sooner than in susceptible varieties, and Hori suggested that the early death of the suscept cells inhibited the fungus. Hori described the production of small spots on all plants in which penetration occurred but in which the fungus did not develop further. Small spots or flecks in resistant varieties have been mentioned also by others (1, 2, 21, 22, 23).

Müller and his coworkers, largely as a result of work with tubers, have developed the theory that the more rapid the necrotic reaction of the invaded potato cell, the greater is the resistance of the plant as a whole (13, 16, 17). In the early phases of this work, Meyer (13) observed that browning occurred in the protoplasm and cell walls of tubers of both susceptible and resistant varieties, but that it occurred more rapidly in resistant than in susceptible varieties. The development of the brown color was thought to be preceded by the formation of substances inhibitory to fungal growth. The extent of development of the fungus, therefore, was related to the speed of formation of the inhibitory substances and of the necrotic reactions. It was suggested that the substances were formed by the cells of the suscept in response to some fungal product. Thus, according to this theory, resistant cells are more sensitive to the fungal product and produce the inhibiting substances faster than susceptible cells. As a result, in resistant tissue a barrier is formed through which the fungus cannot advance, whereas in susceptible varieties the fungus passes through the infected tissue before the inhibiting compounds are formed.

The object of this investigation was to study such interactions in leaves and to compare the progress of the pathogen and response of the suscept, at given time intervals, in susceptible plants with those in resistant plants. The resistant potato varieties used were progeny of crosses between *S. demissum* and *S. tuberosum*. Several races of the pathogen were employed.

In a paper published after the completion of the present investigation, Pristou and Gallegly (21) reported on histological studies with leaflets of the variety Kennebec, upon which are formed small necrotic flecks after inoculation with race 0 of the fungus. They found that penetration occurred in the same manner as in the susceptible variety Cobbler and that a hypha developed in the penetrated epidermal cell. They were unable to detect any mycelial advance from the epidermal cell, although sections

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of leaflets that had been kept at 20°C showed necrosis of cells below the penetrated epidermal cell 24–48 hours following inoculation. Mycelium was not found in the necrotic tissue of the resistant leaflets nor in the surrounding healthy tissue 72 hours after inoculation. It was concluded that disintegration of the mycelium occurred while the mycelium was confined to the epidermal cell.

MATERIALS AND METHODS.—All genotypes of the potato and all races of the fungus that were used were supplied by Professor L. C. Peterson, Cornell University, and Professor W. R. Mills, Pennsylvania State University. The following are the varietal designations of the plants and their genotypes (3):

Variety or Selection	Genotype
Green Mountian	r
Essex	R1
FOL-7	R2
3WN-4	R4
3WM-19	R1R4
DFR-4	R1R1
3RD-5	R2R2

Inoculations were made on young potted plants. Plants of similar size and age were used when possible. Inoculations involving any given combination of plant and fungus were repeated until it was felt that an accurate picture of the typical responses had been obtained. Fungus races 0, 1, 2, and 1,4 were used for the study (3). These were cultured on lima-bean agar. The cultures were transferred every 7 days and were kept at 18°C.

Inoculation and incubation.—Standardized procedures for inoculation and incubation were used in order that the different pathogen-suscept combinations might be compared at given time intervals. Sporangiophores from 10-day-old slant cultures of the fungus were scraped with a needle into a small amount of glass-distilled water that had been stored at 12°C. The spore suspension was then kept at 12°C for 3 hours to promote swarmspore formation. After 3 hours the swarmspore suspension was transferred to a hand atomizer and sprayed on the lower surfaces of all leaves of the test plants until they were covered with the liquid.

The plants were incubated in a lighted chamber maintained at 18°C. Plastic bags were placed over the plants for 17–18 hours to provide high humidity. It was found that the ultimate symptom development at this temperature was the same as that in plants held at slightly higher temperatures in the greenhouse. The symptoms developed more slowly, however, at the lower temperature.

Preparation of material for histological observation.—Most of the data were obtained from a study of leaflets that were cleared and examined whole, as this method of preparation allowed the most rapid and thorough examination of the material of any method used. The technique for preparing the whole leaflets was adapted from that of Paddock (19). The leaflets were cleared in a Carnoy's solution (2 parts absolute alcohol and 1 part acetic acid) and were then stained in a mixture of lactophenol and aniline blue. The stained leaflets were mounted in clear lactophenol.

Sections were cut from leaves at times during the study. Leaf tissue fixed in either formalin-aceto-alcohol (FAA) or osmic acid was embedded in paraffin after dehydration with tertiary butyl alcohol in the usual manner. Microtome sections (5–20μ) were cut and then stained with crystal violet and orange G. Fresh material was sectioned on the freezing microtome and by hand, and the sections were stained with acid fuchsin in lactophenol. The sections were examined with the ordinary microscope and with the phase contrast microscope.

EXPERIMENTAL RESULTS.—The reactions (in terms of resistance or susceptibility) of potato genotypes r, R1, R4, and R1R4 to races 0, 1, 4, and 1,4 of the pathogen are known (3). The reactions for all possible combinations of these genotypes and races may be summarized as follows:

	Race			
Genotype	0	1	4	1,4
r	**S**	**S**	**S**	**S**
R1	—	**S**	—	**S**
R4	—	—	**S**	**S**
R1R4	—	—	—	**S**

In this summary, S denotes susceptibility and — denotes resistance. The reactions selected for histological study are in bold face type. In all cases where such studies were made, the leaves were examined macroscopically for the symptoms typical of susceptible plants or for the necrotic flecking typical of resistant plants. If histological examinations were made of only 1 or 2 genotypes inoculated with a given race, macroscopic observations were made also of other genotypes inoculated with the same race of the pathogen.

Observations of susceptible plants.—Macroscopic symptoms usually became evident as small flecks about 48 hours following inoculation. These flecks enlarged rapidly and by the third or fourth day following inoculation were large spreading lesions. If conditions were humid, sporangiophores often could be found at the edges of the lesions. Histological observations were made of leaflets harvested at intervals during the first 48 hours following inoculation.

1) *First 10 hours.*—On whole leaflets, the outlines of the empty swarmspores and appressoria could be seen attached over the penetration pegs. Branched and unbranched primary hyphae[2] (stained blue) could be found in the penetrated epidermal cells. There was no visible response of the suscept.

2) *From 17 to 20 hours.*—Little or no response was evident in the penetrated epidermal cells of the suscept, although many penetrated cells were seen, as was the case in samples taken after 10 hours. Occasionally a

[2] The words "primary hypha" denote the first hypha that develops from the penetration peg. As has been observed many times (7, 26) this first hypha usually forms within an epidermal cell where it may branch several times. The words "secondary hyphae" denote intercellular hyphae that develop from the primary hypha.

slight thickening of the walls (Fig. 1) and sometimes also a slight browning of the epidermal cells was observed. The primary hyphae stained blue. In plants of genotype r inoculated with race 0 and in plants of genotypes r and R1 inoculated with race 1, mycelium could be found ramifying into tissue several cells from the site of penetration.

3) *From 26 to 28 hours.*—In most instances the primary hyphae no longer stained blue but appeared clear or hyaline. The penetrated epidermal cells had very thick dark walls (Fig. 4) and were usually a light brown. Occasionally a penetrated cell was dark brown and the contents had a granular appearance. Secondary hyphae, stained blue, could be followed through the intercellular spaces of the mesophyll to a distance equivalent to 3–4 cells from the site of penetration. In some instances there was a slight browning of a mesophyll cell located directly beneath the penetrated epidermal cell. This browning was restricted usually to regions of the cell in contact with the secondary hyphae.

The genotypes differed with respect to the speed with which the necrotic responses of the cells developed. The responding mesophyll cells of plants of genotype R1R4 inoculated with race 1,4 appeared to be browner than those of the plants of genotype R1 inoculated with the same race.

4) *From 42 to 49 hours.*—The penetrated epidermal cell and 1 or 2 of the neighboring epidermal cells often were clear. The remains of the primary hypha usually could be seen. Several mesophyll cells directly under a penetrated epidermal cell showed an acute response. The cellular contents were clumped so that no differentiation among chloroplasts or between chloroplast and nucleus was possible. These responding cells still appeared to be joined to each other at the middle lamellae (Fig. 3). The secondary hyphae were hyaline in the region of the browning mesophyll cells (an area of 2 to 4 cells). Advance mycelium radiating from the lesion was stained blue. Occasionally a few deep-lying spongy mesophyll cells that were separated from the area originally invaded were found to be light brown.

Observations of resistant plants.—Small necrotic flecks were usually visible macroscopically 48 hours following inoculation, as in the susceptible varieties. These flecks, however, did not enlarge or change in appearance. No sporangiophores were produced on leaves of resistant plants. Histological observations were made of leaflets harvested at the same intervals as in the study of susceptible plants.

1) *First 10 hours.*—Penetrated epidermal cells were found, as in susceptible plants. The penetrated cells contained primary hyphae in various stages of differentiation. There was no suscept response.

2) *From 17 to 20 hours.*—The response of the penetrated epidermal cells often was more acute than that of epidermal cells of susceptible varieties (Fig. 2). In many cases the primary hyphae had become clear. Sometimes the mycelium was restricted to the epidermal cell (Fig. 2), but often a secondary hypha had grown for a short distance between spongy mesophyll cells. In such instances there was a slight browning of parts of the mesophyll cells. The secondary hyphae had stained blue or had become clear. In no instance was there wide ramification of the secondary hyphae.

The genotypes differed in the rapidity with which the necrotic response developed. The response of genotype R1 to race 0 was slower than that of genotype R1R4 in all 3 instances in which genotype R1R4 was resistant to the race of the pathogen used.

3) *From 26 to 28 hours.*—The penetrated epidermal cells were dark brown and contained clear primary hyphae (Fig. 5). Where short secondary hyphae had formed, the mesophyll cells near these hyphae were brown and the contents clumped (Fig. 6). The intercellular hyphae often had become cleared but occasionally had stained a dark green.

4) *From 42 to 49 hours.*—The lesions resembled those of resistant plants after 26–28 hours. The penetrated epidermal cells were either brown and granular as before or they had become cleared and their outlines were difficult to distinguish. It was possible at times to see cleared remains of primary hypha in such cells. Spongy mesophyll cells near secondary hyphae were brown, and their contents were clumped as in

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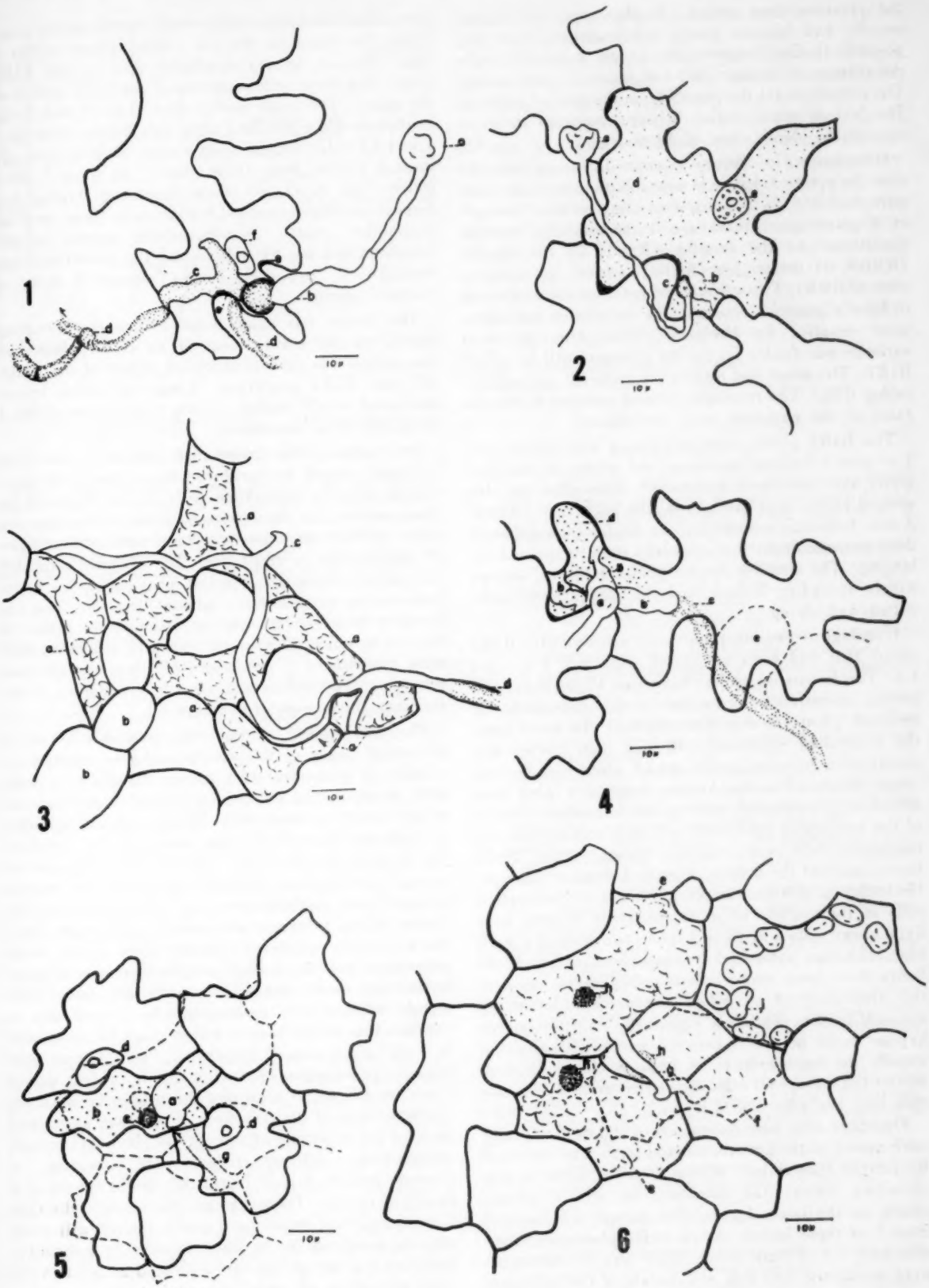
FIG. 1–6. Camera lucida drawings of whole-leaf preparations, illustrating responses of susceptible and resistant plants. —Fig. 1. Susceptible reaction (race 1—genotype r), 18 hours following inoculation. Empty swarmspore (a), germ tube, and appressorium (b) were attached over the penetration peg. Branched primary hypha (c) stained blue. Secondary hyphae (d) could be followed between mesophyll cells. The only suscept response was a slight thickening (e) of the epidermal cell wall near the site of penetration. (f, nucleus).—Fig. 2. Resistant reaction (race 4—genotype R1R4), 18 hours. Swarmspore (a) and germ tube were hyaline, and also the primary hypha (b). Appressorium (c), however, stained blue. Penetrated epidermal cell (d) was brown and had thickened walls. (e, nucleus).—Fig. 3. Susceptible reaction (race 1—genotype R1), 49 hours. Several cells (a) of the first mesophyll layer were brown and shrunken, and their contents had become clumped. These were surrounded by cells that showed no visible response (b). Hyaline mycelium (c) could be found in the area. Hyphae that stained blue (d) were between nearby mesophyll cells.—Fig. 4. Susceptible reaction (race 1—genotype R1), 28 hours. Appressorium (a) and branched primary hypha (b) were hyaline. Secondary hypha (c) stained blue. The thickened walls and brown color of the epidermal cell were limited to an area (d) near that of penetration. (e, mesophyll cell).—Fig. 5. Resistant reaction (race 0—genotype R1), 26 hours. Primary hypha (a) was hyaline. Penetrated epidermal cell (b) was brown and granular, and the nucleus (c) was smaller than the nuclei (d) of other epidermal cells. (g, secondary hypha).—Fig. 6. The same lesion as that of Figure 4, showing 2 responding cells (e) of the first mesophyll layer and 1 cell (f) of the second layer. Secondary hyphae (g) were hyaline. (h, nuclei of responding mesophyll cells; i, chloroplasts of nonresponding mesophyll cell).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the previous time period. At this time all hyphae usually had become clear, but occasionally it was possible to find between the brown mesophyll cells the remains of hyphae that had stained a dark green. The mycelium did not ramify from the area of a lesion. The lesions were similar in appearance to those of susceptible plants after 42–49 hours.

Observations of duplex plants.—It is now thought that the potato behaves in many instances as an autotetraploid (12, 25). Thus, depending on the "dosage" of a given gene, there are 4 theoretically possible conditions: 1) the simplex (Rrrr), 2) the duplex (RRrr), 3) the triplex (RRRr), and 4) the quadruplex (RRRR). Two varieties of potatoes, each believed to have a gene for resistance in the duplex condition, were supplied by Professor Mills. One of these varieties was duplex for the R1 gene and will be called R1R1. The other was duplex for gene R2 and will be called R2R2. The reactions of these potatoes to several races of the pathogen were investigated.

The R1R1 plants were inoculated with either race 1 or race 1,4 of the pathogen, and plants of genotype R2R2 were inoculated with race 2. Susceptible simplex control plants were inoculated with each race. Within 3 days following inoculation, the duplex plants showed dark necrotic flecks that appeared to have stopped enlarging. The simplex control plants, however, showed widely spreading lesions, and the plants were badly wilted and dying.

Histological examinations were made of the R1R1 plants that had been inoculated with race 1 or race 1,4. The lesions of leaflets examined 17–20 hours following inoculation resembled those described for resistant plants at this time interval. In most cases the penetrated epidermal cell was light brown and contained a primary hypha, which often had become clear. Short secondary hyphae sometimes were seen between the mesophyll cells in the immediate vicinity of the penetrated epidermal cell, and occasionally the mesophyll cells were slightly brown. After 26–28 hours, many of the lesions resembled those of susceptible leaflets at this time interval. Often 1 or 2 mesophyll cells in the vicinity of short secondary hyphae were light brown. Most of these hyphae had stained a dark blue, although some had become clear. After 42–49 hours there were many lesions unlike those found at this time interval on either the susceptible or the resistant leaflets previously examined. Short secondary hyphae could be found between mesophyll cells, but usually the hyphae had not become clear. In such lesions the mesophyll cells showed no greater response than they had after 26–28 hours.

Five days after inoculation, all of the duplex plants were moved to the greenhouse and were retained there for further observation. Within 3 weeks, a few widely spreading lesions had developed on all the plants, chiefly on the lower leaves. The fungus was isolated from 1 of these lesions on an R1R1 plant inoculated with race 1,4. Plants of the R1R1 and R1 genotypes were inoculated with this new isolate of the pathogen. Spreading lesions, typical of those on susceptible genotypes, developed on the R1 control plants within 4 days. Similar lesions developed also on the R1R1 plants, but these were restricted to the lower leaflets of the plants. The upper leaflets showed small dark flecks similar to those obtained after inoculation with races 1 and 1,4. Histological studies were made of centrally located leaflets from these plants. In these leaflets, lesions that developed where secondary hyphae had formed usually resembled very closely those seen on susceptible plants. Sometimes the growth of the mycelium did not extend beyond the penetrated epidermal cell, and such lesions resembled those of resistant plants.

The fungus was again isolated from the spreading lesions on the lower leaves of an R1R1 plant, and this isolate was used to inoculate plants of the R1R1, R1, and R1R4 genotypes. Large spreading lesions developed on all leaflets of the 3 genotypes within 4 days following inoculation.

Discussion.—The theory that substances inhibitory to fungal growth are produced in resistant cells more rapidly than in susceptible cells is not disproved by these studies, but the results do indicate that the rate of the necrotic responses is not a dependable indicator of susceptiblity or resistance. The necrotic responses of resistant plants developed at most only a few hours before those of susceptible plants (Fig. 7). The differences between resistant and susceptible plants in the rate at which the necrotic responses developed were most pronounced if genotype R1R4 was the resistant genotype. The differences were less apparent if the resistant plants were of genotype R1.

The development of the necrotic responses or of any other phase of the suscept-pathogen interaction, whether in a resistant or a susceptible plant, is probably under partial control of factors other than the major genes for resistance. Degrees of susceptibility or resistance have long been noted among plants of the r genotype (4, 5, 11, 18, 23, 24). Degrees of attack have also been reported for clones that contain a major gene for resistance, an indication that such clones belong to one of the resistant genotypes (10). Black (2) has suggested that the effect of the major genes may not be absolute and has spoken of other factors that modify the reaction as "minor genes". He recognized that such genes might be present also in varieties that do not possess major genes for resistance. If such "minor genes" are exerting an influence over the suscept-pathogen interactions, it is evident that it might be difficult to draw any sharp lines between the characteristics of the responses of resistant plants and those of the responses of susceptible plants. If enough genotypically different plants were examined, a diagram such as Figure 7 probably would consist of a family of curves. There was an indication in the data that in resistant reactions a given variety will react with the same rapidity to different races. In susceptible reactions a given variety also appeared to react in the same manner to all races pathogenic to it.

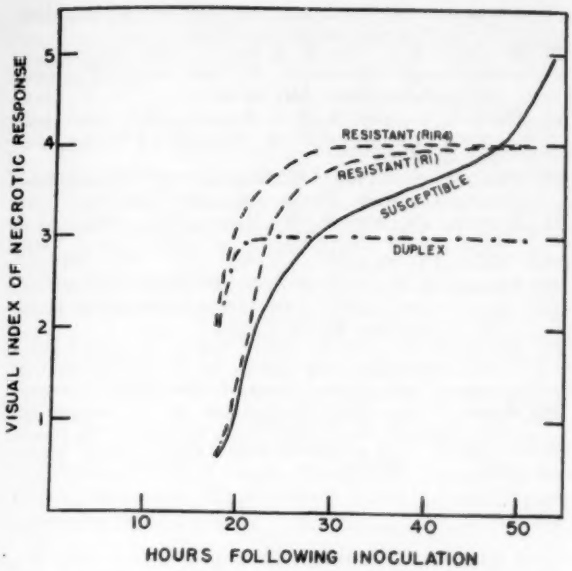

FIG. 7. Diagrammatic representation of the rate of the necrotic responses in a single lesion in different genotypes as determined from the present histological study. The index numbers along the vertical axis represent different stages in the necrotic response. 1—Browning of the wall of the penetrated epidermal cell. 2—Browning of the whole penetrated epidermal cell. 2—Browning (light) of 1 or a few mesophyll cells. 4—Browning (dark) of 1 or a few mesophyll cells and clumping of the contents. 5—Browning of many mesophyll cells.

When plants that contained 2 resistance genes of the same kind were inoculated with races pathogenic to plants with but a single gene of that kind, they developed after 4 days small necrotic flecks similar to those on resistant plants. This indicated that these duplex plants should be considered resistant. The histological examinations of the lesions of the duplex R1R1 plants inoculated with race 1,4 showed that the first stages in the necrotic response of the penetrated cell and the primary hypha occurred quickly (as in resistant reactions of genotype R1R4), but that the later stages of the necrosis of suscept and pathogen developed at a rate that was slower than that in susceptible plants (Fig. 7). The pathogen was able to make only limited growth, however, and the hyphae in most instances were confined to the area of the lesion.

An explanation of the observed reactions in the duplex plants will have to await more information about the mechanisms involved in susceptibility and resistance. It is apparent that in the duplex plants of genotype R1R1 the presence of the second R1 gene in the plants resulted in the development of a milieu unfavorable for the growth of a race of the fungus able to develop extensively in plants that contain only one of the R1 genes. This race, however, appeared to be less sensitive to the effects of the second gene than is a nonpathogenic race to the effects of a single resistance gene. The pathogen survived for a longer time in the duplex plants than is usual for nonpathogenic races in resistant plants.

The symptom picture and the histological data showed that the culture of the fungus isolated after the first passage through the R1R1 (duplex) plant was more pathogenic to the plant than was the original culture. The isolate obtained from the second passage appeared to be still more pathogenic. This penomenon does not seem to be different from the instances already reported in which the pathogenicity of a culture was changed by means of passages through resistant plants (14, 15, 20, 22, 23). Probably such passages through plants that differ from others in the number of resistance genes of a given kind will provide a means of finding still more isolates of the fungus that differ from other isolates in pathogenicity.

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POINSETTIA ROOT ROT [1]

John R. Keller and James B. Shanks

SUMMARY

There are 2 phases of root rot in greenhouse-grown poinsettias. The first appears when cuttings are rooted and is caused chiefly by species of *Rhizoctonia* and *Pythium*. The second appears as a late season rot just before the plants mature. In older plants, *Thielaviopsis basicola* is the primary pathogen, but root injury is increased when Rhizoctonia and Pythium are also present. Isolates of *T. basicola* were shown to vary in pathogenicity when tested on poinsettia and tobacco. Isolates from poinsettia caused root rot of that host but not of tobacco, whereas those from tobacco did not injure poinsettia. Use of soil with a pH below 5.0 prevented injury from *T. basicola*. A greenhouse night temperature of 70°F delayed, but did not prevent, Thielaviopsis root rot, whereas 50°F was very favorable for its development. Root rot was favored by high soil moisture. Among 11 poinsettia varieties tested for reaction to root rot fungi, Pink and St. Louis were the most susceptible and the Indianapolis Reds the least susceptible.

Root and stem rots have been of increasing importance in poinsettia production. In recent years growers in all sections of the United States have lost entire crops. As a result, some growers have abandoned poinsettias. Control measures for these rots have consisted largely of sanitation practices and use of a ferbam (ferric dimethyldithiocarbamate) coating of the cuttings. Where these measures have been applied diligently, there has been fair disease control; however, many growers still receive infected stock plants.

In the past, growers produced their own stock plants, but more recently it has been a practice to buy them from California. This has the advantage of providing vigorous field-grown plants. Such plants are received about late March, and cuttings are made in the summer or early fall. These are rooted and potted and then grown in greenhouses where the night temperature is maintained at 55–65°F.

There are 2 periods in poinsettia culture when rots are most prevalent—soon after cuttings are potted and just before the plants have matured for Christmas sale. *Rhizoctonia solani* Kühn (7, 9, 17, 26), *Pythium debaryanum* Hesse, and *P. ultimum* Trow (17, 18, 20, 22) have been cited as the causes of root and stem rots of cuttings and potted plants of poinsettia. Stem lesions usually appear at the soil line and become dark brown and water-soaked in appearance. Under favorable conditions they increase in size, and the cuttings wilt and die. A root rot affecting poinsettias was described by Dimock (8) as being caused by

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Thielaviopsis basicola (Berk. & Br.) Ferr. and a species of *Pythium*. Tilford (24) noticed an association between *T. basicola* and a root rot of poinsettia stock plants but did not prove that this fungus was a cause of the disease.

Root rot of poinsettia is characterized by stunted plants, excessive leaf drop, and badly rotted roots. Occasionally, leaves and flower bracts are distorted. Symptoms are influenced by the time of infection. Cuttings that are infected soon after potting may suffer nearly complete loss of roots and leaves, whereas those infected near maturity may have only mild symptoms. One of the most characteristic symptoms of infection is an inward rolling of the leaves before they turn yellow and abscise. Infection is confined to the roots and the belowground portion of the stem. Infected roots turn brown-black and decay. Black rough longitudinal cracks frequently occur on the belowground part of the stem. Cuttings made from infected stock do not carry the fungus.

T. basicola has caused root rot of tobacco for years and is reported to have a host range of more than 100 species (12). Johnson and Hartman (13) found no evidence of different pathogenic strains of *T. basicola*. Later research from Europe, however, showed that physiologic strains do occur. Tiddens (25) found that isolates from primula, tobacco, and poinsettia were most pathogenic on the host from which they were isolated. Sattler (21) obtained similar results with isolates from the United States and Germany tested on tobacco, beans, and lupine. Allison (1) observed specialization of several isolates on tobacco.

Clinton (5) noticed a correlation between the use of large amounts of potash and the incidence of root rot damage to tobacco. A year later, Briggs (3) reported that alkaline soil markedly favored root rot, whereas in acid-treated soil, roots were entirely free of the fungus. The same effect has been reported by other workers (4, 13, 21). Anderson, Osmun, and Doran (2) showed that extensive losses from black root rot occur in soils with a pH above 5.9, whereas practically no losses occur below pH 5.6 except in cold soils. They further showed that in pure culture, *T. basicola* grew very well on Czapek's agar above pH 6.05, grew more slowly at about pH 5.35, and ceased to grow at pH 3.05. Johnson and Hartman (13) reported that optimum, maximum, and minimum temperatures for growth in pure culture were 30, 34–37, and 7–8°C, respectively. They noticed, however, that the optimum for tobacco root rot ranged between 17–23° with no infection at 31–32°. Sattler (21) observed a similar response with beans and lupine. The amount of tobacco root injury from *T. basicola* also has been found to be directly proportional to the amount of soil moisture (2, 5, 10, 13, 21).

T. basicola is easily recognized in infected host tissues by the characteristic dark brown to black, thick-walled, barrel-shaped chlamydospores (Fig. 1) that constitute the second stage of spore production (5, 10, 28). Hyaline endoconidia comprise the first stage and are formed in the elongate terminal cell of the thin-walled endoconidiophores. Chlamydospores are borne on the same hyphae as the endoconidiophores or on any other portion of the mycelium. They may be single, but usually they are found in chains. Eventually they break up into individual cylindrical cells, each of which is capable of germination. Some writers (15, 16, 23) have concluded that the perithecial stage, *Thielavia basicola* Zopf, has no genetic relationship to the endoconidia and chlamydospore stages of the imperfect fungus *Thielaviopsis basicola* and that Thielavia exists only in association with Thielaviopsis.

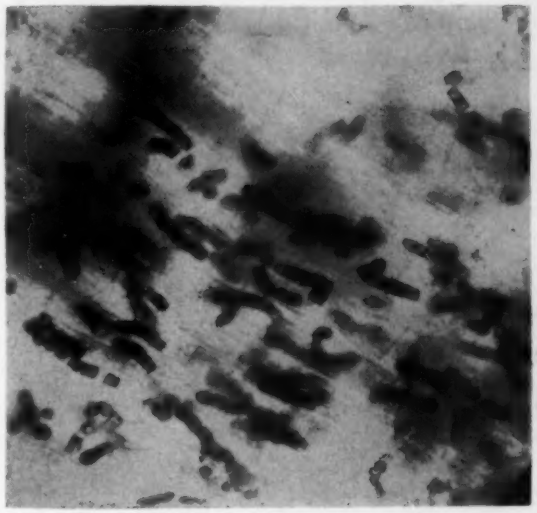

FIG. 1. Chlamydospores of *Thielaviopsis basicola* in poinsettia root tissue.

The objective of the present study was to obtain information on 1) the roles of Thielaviopsis, Rhizoctonia, and Pythium in the poinsettia root rot problem; 2) the relative pathogenicity of various isolates of *T. basicola*; 3) the influences of temperature, pH, and moisture level on disease incidence; and 4) the possibility of at least partial resistance in certain poinsettia varieties.

RELATIVE IMPORTANCE OF DIFFERENT FUNGI, ALONE AND IN COMBINATION, AND THE EFFECT OF SOIL MOISTURE ON ROOT ROT.—*Thielaviopsis basicola* was isolated from poinsettias in Maryland by the carrot-disk technique described by Yarwood (27). A culture of *Rhizoctonia* sp. and 2 isolates of Pythium were obtained from poinsettias in New York State.[2] Rhizoctonia and *T. basicola* were cultured on sterilized oats for approximately 1 month. About ¾ cup of inoculum was used in each lot of soil where required. The Pythium isolates were cultured on plates of potato-dextrose agar, and each mycelial mat was transferred to a Waring Blendor in 250 ml of water

[2] Obtained from Dr. A. W. Dimock of Cornell University. These have never been identified as to species, but probably are close to *P. ultimum* or *P. debaryanum*.

TABLE 1.—*Root and top weights of poinsettia varieties Ruth Ecke and Barbara Ecke Supreme grown in soil infested with different fungi, alone and in combinations*

Treatment	Ruth Ecke[a]		Barbara Ecke Supreme[a]	
	Top wt.	Root wt.	Top wt.	Root wt.
	g	g	g	g
Noninfested	1027	146	1044	129
T. basicola	663	90	506	62
Rhizoctonia	958	120	1082	130
Pythium sp. A[b]	845	91	1066	123
Pythium sp. B[c]	1140	132	1175	142
T. basicola plus Rhizoctonia	574	73	543	66
T. basicola plus Pythium sp. B	566	69	632	70
Rhizoctonia plus Pythium sp. B	1144	153	1156	119
T. basicola plus Rhizoctonia plus Pythium sp. B	497	64	599	49

[a] LSD at 1 per cent level: tops, 106; roots, 25.
[b] Isolated at Ithaca, New York.
[c] Isolated at Farmingdale, New York.

and triturated for 30 seconds. The suspension was next diluted in a liter of water and sprinkled on the previously moistened soil. Three plants of the variety Ruth Ecke and 3 of Barbara Ecke Supreme were planted in each of 108 six-in. pans of clay-loam soil (pH 6.6). Twelve pans were left as noninfested controls, and groups of 12 were infested as shown in Table 1. Each of these groups was divided into 3 lots. One lot (watered normally) was watered 16 times in 35 days, another (low water) was watered 9 times, and the other (excess water) was watered 29 times in 35 days. The night temperature was maintained at 60°F. The experiment was begun in early November of 1953 and the tops and roots were weighed separately 6 weeks later. All soil was carefully removed by washing and the roots blotted prior to weighing. Within limits, the top growth was proportional to the vigor of the root system (Table 1), and for this reason top weights were considered to be a fairly accurate indication of the extent of root rot.

TABLE 2.—*Top and root weights of poinsettia varieties Ruth Ecke and Barbara Ecke Supreme grown in soil infested with different fungi and maintained at 3 moisture levels*[a]

Moisture level	Noninfested	*T. basicola*	Rhizoctonia	Pythium sp. A	All 3 fungi
	Top weight (g)[b]				
Low	542	391	505	582	325
Normal	708	402	669	644	392
High	821	376	866	685	379
	Root weight (g)[c]				
Low	93.9	59.3	62.7	76.9	43.2
Normal	72.9	50.0	76.8	68.1	33.9
High	108.9	42.9	110.8	68.7	35.8

[a] Data are the averages for 2 varieties.
[b] LSD at 1 per cent level is 86.6.
[c] LSD at 1 per cent level is 20.4.

T. basicola was the most pathogenic of the fungi tested (Table 1). *Pythium* species A and Rhizoctonia damaged Ruth Ecke plants but not Barbara Ecke Supreme. *Pythium* species B caused no injury to either variety even when used in combination with Rhizoctania. When *Pythium* species B and Rhizoctonia were combined with *T. basicola*, however, extensive damage resulted. Symptoms appeared sooner with the combinations of *T. basicola* with Rhizoctonia or *Pythium* species B, or with both (Fig. 2, A), than in the other treatments.

High moisture levels favored the disease development (Table 2). At the high water level, partial to complete defoliation occurred with *T. basicola*, and with combinations including *T. basicola*, several weeks before defoliation was serious at the low and normal water levels (Fig. 2, B). Root weights tended to reflect the increased injury by *Pythium* species A at higher moisture levels more than did top weights. Apparently, the aboveground portion had responded to the benefits of the increased water level before root rot was sufficient to check growth. The fungi were reisolated from representative poinsettia roots at the completion of the experiment.

EFFECT OF 4 ISOLATES OF T. BASICOLA AT 3 pH

FIG. 2. Foliage symptoms and root development of poinsettia plants grown in soil infested with various fungi. A) Foliage symptoms in variety Ruth Ecke. Left to right: *T. basicola*, Rhizoctonia, noninfested control, *T. basicola* plus Rhizoctonia, and *T. basicola* plus Pythium B. B) Plants of variety Ruth Ecke grown in soil infested with *T. basicola* and maintained at (left to right) low, normal, and excess moisture levels. C) Plants of variety Ruth Ecke grown in soil infested with different isolates of *T. basicola*. Left to right: Noninfested control, Maryland isolate from poinsettia, and a brown isolate from tobacco. D) Plants of the variety Barbara Ecke Supreme grown in soil infested with *T. basicola* and adjusted to pH levels of (left to right) 4.8, 6.2, and 7.3. E) Varietal reaction of 5 varieties of poinsettia 5 weeks after transplanting to soil infested with *T. basicola*. Left to right: Ruth Ecke, Pink, Ecke White, Indianapolis Red, and St. Louis. F) Roots of the variety Barbara Ecke Supreme, 4 weeks after transplanting to soil infested with *T. basicola*, showing effects of soil pH on root rot. Top to bottom: pH 7.3, pH 6.2, and pH 4.8. G) Roots of the variety Ruth Ecke 4 weeks after transplanting to soil infested with *T. basicola*, showing effects of temperature on root rot. Top to bottom: 70°, 60°, and 50° F.

A
B
C
D
E
F
G

TABLE 3.—*Top and root weights*[a] *of poinsettia plants grown at different temperatures in soil at different pH levels infested with different isolates of T. basicola*

Temperature	Initial pH	Noninfested soil		Soil infested with *T. basicola* from: Poinsettia				Tobacco				Totals for pH	
				Maryland		New York		Brown		Gray			
		Top	Root	Top	Root	Top	Root	Top	Root	Top	Root	Top	Root
°F		g	g	g	g	g	g	g	g	g	g	g	g
50°	4.8	502	62	553	64	483	57	493	63	480	54	2491	300
	6.3	583	73	184	27	323	38	586	68	566	67	2242	274
	7.3	541	72	190	30	297	39	517	70	483	53	2028	267
Totals for 50°		1626	207	907	121	1104	134	1595	202	1528	177	6760	841
60°	4.8	619	103	698	94	663	95	557	91	586	82	3123	465
	6.3	715	109	224	36	508	66	699	99	751	99	2897	409
	7.3	782	122	268	37	497	64	634	93	589	88	2770	404
Totals for 60°		2116	334	1190	167	1668	225	1889	283	1926	269	8788	1278
70°	4.8	657	100	824	105	679	86	646	89	589	88	3395	468
	6.3	796	104	467	54	675	76	855	107	918	105	3711	446
	7.3	753	102	507	60	707	77	787	101	859	111	3613	451
Totals for 70°		2206	306	1798	219	2061	239	2287	297	2365	304	10717	1365
Totals for all temperatures		5947	847	3895	507	4833	598	5772	782	5819	750		

[a] LSD at 1 per cent level for top weight and root weight, respectively: isolates, 384 and 73; temperature and pH, 495 and 94; isolate × temperature and isolate × pH, 222 and 43; temperature × pH, 286 and 54; isolate × pH × temperature, 128 and 24.

LEVELS AND 3 TEMPERATURES ON POINSETTIA VARIETIES AND ON TOBACCO.—A steam-sterilized mixture (pH 5.3) of equal parts of acid peatmoss[3] and loam was adjusted to pH 4.8, 6.3, or 7.3 by treatment with either hydrated lime or sulfuric acid and sulfur. The sulfuric acid, diluted with a large volume of water, was used to reduce the pH level immediately. It was assumed that gradual oxidation of the sulfur to sulfuric acid would maintain a low pH throughout the course of the experiment. Two isolates of *T. basicola* were from poinsettias grown in Maryland and New York[2]; the other 2 isolates, the brown and the gray[4], were from tobacco. All 4 isolates were cultured on sterilized oats for about 1 month before use. The 11 poinsettia varieties used were Albert Ecke, Barbara Ecke Supreme, Ecke White, Improved Albert Ecke, Improved Indianapolis Red, Indianapolis Red, Oakleaf, Mrs. Paul Ecke, Pink, Ruth Ecke, and St. Louis[5]. The tobacco variety No. 402, known to be susceptible to *T. basicola*, was used. The poinsettias and tobacco were planted in 4-in. pots between October 26 and 30, 1953. Three established plants in 2¼-in. pots and 3 rooted cuttings of each poinsettia variety were used in each of the treatments formed by the combinations of isolates, soil pH, and temperature. Eight tobacco plants were used in each treatment. Approximately 500 ml of inoculum, prepared as previously described, was used for each 4 bushels of soil. In December and January, data were recorded on stem and bract lengths and root and top weights. The soil pH levels were checked after approximately 2 weeks and again 4 weeks later. The averages for the low, medium, and high pH levels after 6 weeks were 4.8, 6.2, and 7.1, respectively. The night temperatures in separate greenhouse sections maintained at or near 50, 60, and 70°F remained within 2° of the temperature desired. Root and top-weight data were obtained as described previously.

Evidence of the existence of pathogenic strains of *T. basicola* was obtained (Tables 3 and 4). Isolates from poinsettia severely damaged poinsettias but not tobacco (Fig. 2, C). The brown isolate from tobacco severely damaged tobacco but not poinsettia. The Maryland isolate was much more virulent than the New York isolate. By mid-December, complete defoliation resulted with most poinsettia varieties infected with the Maryland isolate, whereas the New York isolate caused only partial defoliation. Effects of both isolates were influenced by variety, temperature, and soil pH. Use of soil with a pH below 5 prevented nearly all infection by *T. basicola* (Fig. 2, D, F). Top weights of poinsettias in the check pots (Table 3) were reduced slightly in acid soil although this was not noticeable by casual observation of the plants during the experiment. Bract size and color were the same at all pH levels. Both the low and the high pH levels depressed tobacco growth markedly (Table 4), the low more so than the high. In the acid soil, however, tobacco roots were free of infection. At 70°F, root rot development was delayed for 7 weeks, then there was a considerable loss of roots and leaves with the Maryland isolate, but not with the New York isolate (Fig. 2, G and Fig. 3). At this temperature the bract color was not as good as at 50° or 60°. Also,

[3] Premier brand: German source.

[4] Obtained from Dr. O. D. Morgan, Jr., University of Maryland, who originally obtained them from Dr. R. H. Stover.

[5] Stock plants of 11 poinsettia varieties were supplied by Mr. Paul Ecke, Encinitas, California.

TABLE 4.—*Top and root weights[a] of poinsettia plants grown at different temperatures in soil at different pH levels infested with different isolates of T. basicola*

Temperature	Initial pH	Noninfested soil		Soil infested with *T. basicola* from: Poinsettia isolate, Maryland		Poinsettia isolate, New York		Tobacco isolate, Brown		Tobacco isolate, Gray		Totals for pH	
		Top	Root	Top	Root	Top	Root	Top	Root	Top	Root	Top	Root
°F		g	g	g	g	g	g	g	g	g	g	g	g
50°	4.8	345	44	426	51	317	39	235	38	328	45	1651	207
	6.3	673	74	688	90	705	93	280	22	564	62	2910	341
	7.3	319	27	569	63	577	64	214	19	497	59	2276	232
Totals for 50°		1337	145	1683	204	1599	197	729	69	1389	166	6737	781
60°	4.8	506	42	522	52	446	25	415	30	485	35	2374	184
	6.3	1061	90	800	81	745	75	366	22	694	64	3666	332
	7.3	793	72	685	71	704	71	316	23	697	63	3195	300
Totals for 60°		2360	204	2007	204	1895	171	1097	75	1876	162	9235	816
70°	4.8	293	28	754	54	482	23	635	28	390	19	2554	152
	6.3	839	139	1131	85	1047	118	716	34	1010	49	4743	425
	7.3	464	41	949	54	650	52	567	28	830	43	3460	218
Totals for 70°		1596	208	2834	193	2179	193	1918	90	2230	111	10757	795
Total for isolates		5293	557	6524	601	5673	561	3744	234	5495	439		

[a] LSD at 1 per cent level for top weight and root weight, respectively: isolates, 683 and 81; temperature and pH, 882 and 93; isolate × temperature and isolate × pH, 397 and 42; temperature × pH, 509 and 54; isolate × pH × temperature, 228 and 24.

if high temperatures are used before the flower buds form, blindness or a delay in flowering combined with excessively long stems may occur. At 50°, the rot was severest, and symptoms appeared in about 2 weeks. This temperature not only favored the disease, but it also repressed poinsettia and tobacco growth. At 60°, symptoms in poinsettias exposed to the Maryland isolate appeared about 3 weeks after the experiment began. The varieties Pink, St. Louis, and Ruth Ecke were the first to show symptoms and these were soon followed by Barbara Ecke Supreme and Oakleaf. Varieties that seemed to have some resistance to the disease were Indianapolis Red, Improved Indianapolis Red, and Albert Ecke (Fig. 2, E). Nevertheless, all poinsettia varieties in soil infested with the Maryland isolate finally became completely defoliated at 50° and 60° at pH levels 6.3 and 7.3.

DISCUSSION. The greatest single factor affecting the development of disease in these experiments was virulence of the isolates used. Perhaps not enough consideration has been given to this in some research. The initial virulence of an organism and its ability to retain that virulence under the influence of environment, host, and cultural conditions is important. It is well known that many pathogenic fungi and bacteria lose virulence rapidly upon culturing. Sattler (21) noted that in agar culture, *Thielaviopsis basicola* decreased in virulence but that virulence could be restored by passage through living plants. Stover (23) found that field isolates of *T. basicola* were made up of 2 types, a brown and a gray. Of these, the brown was the more pathogenic. In culture, avirulent mutants arise that may displace the field types. These cannot compete with the field types in the soil. Stover (23) suggested that many variations in the morphology and pathogenicity of fungi are the result of mutations in growth induced on a nutrient-rich substrate. The marked difference in pathogenicity between the 2 isolates of *T. basicola* from poinsettias possibly is due to the fact that the isolate from New York had been in culture much longer than the Maryland isolate. That changes in *T. basicola* do occur is indicated by the fact that the 2 tobacco isolates were originally highly pathogenic, but after a few years in dry sterile sand, only the brown isolate was weakly pathogenic.

The evidence is good that there are pathogenic strains that attack tobacco and not poinsettia, and *vice versa*. This indicates that the common tobacco field strains of *T. basicola* are not harmful to poinsettias and that field soil used in greenhouse work probably is not a source of fungus introduction unless growers have carelessly dumped old poinsettia soil where it could have contaminated field areas. In view of the wide host range of *T. basicola* (12), it is probable that some pathogenic strains overlap on some hosts and are specific to others. The brown strains of *T. basicola* from poinsettia and tobacco

FIG. 3. Plants of the variety Ruth Ecke, 4 weeks after transplanting to soil infested with *T. basicola*, showing effects of temperature on foliage symptoms. Left to right: 50°, 60°, and 70° F.

were both somewhat pathogenic to geranium. This phenomenon must occur in numerous other cases.

It may not be a coincidence that Rhizoctonia and Pythium commonly are found associated with *T. basicola*. Koch's (14) investigations showed that "numerous other organisms are indisputedly associated with *T. basicola* in black root rot of tobacco." Isolates of Pythium and Rhizoctonia were the chief contributors to root damage and in themselves proved capable of being primary pathogens. According to Koch, the disease picture in the field must include the interaction of several organisms rather than just 1 parasite. On the basis of the authors' experiments, it may be that the presence of *T. basicola* is required before the other organisms are fully pathogenic. When alone, *T. basicola* acts very slowly and is typically restricted to the cortical cells. Roots of tobacco No. 402 infected with the brown strain showed almost no discoloration until a few weeks before the roots were weighed, yet the stunted growth of the plants at the outset showed that there must have been considerable infection by *T. basicola* very soon after the experiments were begun. The presence of Rhizoctonia and Pythium with *T. basicola* hastened the disintegration of infected poinsettia roots. Where combinations of fungi were used, complete leafdrop occurred several weeks earlier than where *T. basicola* was used alone. This is undoubtedly the case in greenhouse soils where there has been no soil sterilization. High water levels and low temperature experienced during late fall are as advantageous to *Pythium* species as to *T. basicola*. In commercial greenhouses, poinsettias have been known to lose their leaves within a week after symptoms appear. Obviously, the roots have been infected for some time previously, but the speed of the leafdrop indicates that there is more than 1 pathogen present. The authors feel that root rot development in poinsettias parallels that of tobacco as discussed by Koch (14).

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FILIPIN, AN ANTIBIOTIC EFFECTIVE AGAINST FUNGI [1]

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SUMMARY

A new antifungal antibiotic, filipin, has been isolated from cultures of *Streptomyces* **filipinensis** n. sp. This compound is an unsaturated substance of the conjugated polyene type. It inhibits a wide variety of fungi, including parasites of plants and animals. Filipin is not highly phytotoxic and gives protection against some seed-rotting fungi as well as against a leaf parasite. It was extracted from the culture filtrate with ether, further purified with petroleum ether, and finally crystallized by trituration with chloroform. Filipin is also readily extracted from the mycelium with methanol or n-butanol. It is nearly insoluble in water, but soluble in methanol, ethanol, n-butanol, pyridine, dimethylformamide, and glacial acetic acid.

Comparatively few antibiotics with antifungal properties have been isolated from cultures of streptomycetes despite the potential importance of such compounds to plant disease control. Many streptomycetes, however, inhibit fungi *in vitro*. The isolation of new fungicides from these microbes could lead not only to the discovery of effective fungicides *per se*, but could also reveal novel structures which might form the basis for further synthetic programs. One streptomycete that was found in a Philippine soil (Negros Occ. 3874) was especially inhibitory to fungi. This organism was chosen for the study that led to the isolation of filipin. A preliminary note on this antibiotic has been published (2).

THE ORGANISM.—Isolate 114-8 possessed characteristics that differentiated this organism from other streptomycetes described in Bergey's Manual of Determinative Bacteriology (1). In addition, this isolate was compared with some of the previously described cultures on the basis of the utilization of various carbon compounds for growth. A sufficient number of differences between isolate 114-8 and the other streptomycetes in Bergey's Manual formed the basis for its description as a new species. This organism is now called *Streptomyces* **filipinensis** n. sp. on the basis of its morphology and physiological responses.

S. filipinensis forms the usual compact substrate mycelium which is light buff in color. The aerial mycelium is white initially and then, as sporulation begins, becomes light grey. The sporophores form spirals that vary from an open to a tightly closed spring-like structure. The spores are round to oval. On Emerson's-agar slants, the organism grows best at 37°C but forms no aerial mycelium at this temperature. Maximum production of aerial mycelium occurs at 23–26°. No growth occurs at 47°. *S. filipinensis* belongs to the chromogenic type in the classification system of Waksman and Henrici (7). A description of growth on a number of media follows:

Nutrient agar at 26°: Very poor growth, light buff, appressed surface mycelium, no aerial mycelium, brown soluble pigment, no distinct color on under side of mycelium, no odor.

North's gelatin agar at 26°: Fair growth, no aerial mycelium, no distinctive color, brown soluble pigment, weak to fair liquefaction of gelatin.

Gelatin stab at 23°: Slow but definite liquefaction, stratiform type, brown soluble pigment.

Asparagine-glycerol agar at 26°: Excellent growth. Aerial mycelium, white turning grey, mycelium shows tendency to grow into medium, slight amount of yellow soluble pigment.

Czapek's sucrose agar at 26°: Excellent growth, cottony aerial mycelium, white turning grey, reverse of colony light yellow, slight amount of yellow soluble pigment. Colorless drops of exudate on mycelium.

Czapek's starch agar at 26°: Excellent growth, velvety aerial mycelium, white turning grey, no soluble pigment, starch hydrolysis weak.

Nutrient broth (stationary culture) at 26°: Slight growth appears on surface, then drops to bottom, brown pigment.

Potato plug at 26°: Good growth, no aerial mycelium, purple to black pigment.

Tyrosinase production: Slight.

Peptone-iron agar: Brown darkening.

Nitrate broth at 26°: None to very weak nitrate formation.

The utilization of various carbon compounds in synthetic media was determined by methods described in a previous publication (4). Inocula for the agar slants were of 3 types: washed mycelia from shaken flasks, spores transferred directly from Emerson's-agar cultures, and soil from soil tube cultures. The results were similar by all these methods.

S. filipinensis grew on media containing any one of the following compounds: xylose, arabinose, fructose, galactose, sucrose, maltose, lactose, raffinose, inulin, mannitol, inositol, sodium acetate, sodium citrate, sodium succinate, dextrose, mannose, starch, dextrin, and glycerol. The compounds that did not support growth were rhamnose, sorbitol, dulcitol, salicin, phenol, *m*-cresol, sodium formate, sodium oxalate,

[1] Accepted for publication June 8, 1955.
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sodium tartrate, and sodium salicylate.

PRODUCTION OF THE ANTIBIOTIC.—*Methods of assay.*—The antibiotic was assayed by 2 procedures: 1) an agar-plate disc method, and 2) a determination of absorption of ultraviolet light. In the disc method, 4-day-old cultures of *Penicillium oxalicum* Thom on V-8 medium[4] were used for the preparation of spore suspensions in sterilized water containing 1:10,000 Vatsol[5]. The suspension was adjusted to 50 per cent light transmission, and 4 ml was then added to 100 ml of cooled trypticase soy agar (Baltimore Biological Laboratory, Baltimore, Md.). For the preparation of the test plates, 20 ml of unseeded trypticase soy agar was poured into sterile petri dishes. After this layer had hardened, 6 ml of the seeded agar was added. In this assay, solutions of filipin in 50 per cent methanol were used to obtain standard curves at a range of 10 to 100 μg/ml.

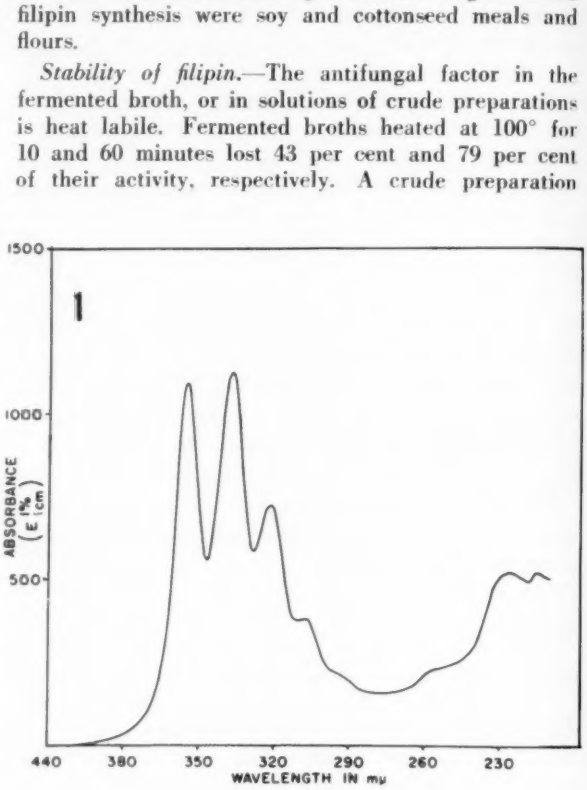
The ultraviolet assay is based on the fact that filipin has the typical polyene absorption spectrum with 3 maxima at 355, 338, and 322 mμ (Fig. 1). The optical densities at the 355- and 338-mμ maxima vary directly with the concentration of filipin in methanol, and a graph of these values becomes a standard curve for assay. The concentration of antibiotic in an unknown sample can be determined by observing the absorption of the unknown sample at these maxima.

For the determination of the filipin content of the fermented broths, a modification of the method was necessary because of the high background absorption by extraneous materials. These background materials increase the intensity of the filipin peaks and give an erroneous assay. Despite this, the difference between the optical densities at a maximum and those at an adjacent minimum is proportional to the concentration of filipin. Since the maxima and minima are very close together, the background absorption is equal at both wave lengths. Its effect on the assay can be nullified by utilizing the difference between the optical density at the 338-mμ maxima and that at the 347-mμ minima as a measure of filipin concentration.

A Beckman Model DU spectrophotometer was used for all assay work. A standard solution of filipin was placed in the cell and the machine set on "check". In this position and at the wave length of the minimum point, 347 mμ, the slit was adjusted until the meter read zero. The wave length was then changed to 338 mμ (a maximum) and the machine turned to the "1" position. The deflection of the needle was returned to zero with the potentiometer, and the optical density was read. This value represented the optical density of the difference between 338 and 347 mμ. If these differences are plotted against concentration, the curve of Figure 2 is obtained. This curve can be used for the assay of culture media containing filipin. The curve follows Beer's Law only up to an optical density difference of 0.55; above this value the slope of Figure 2 cannot be used for filipin assays.

Synthesis in liquid media.—Fermentation studies were carried out in 500-ml shake flasks containing 100 ml of medium on a rotary shaker at 250 rpm at 28°C. Flasks were inoculated with 1 per cent vegetative inoculum grown 3 days in a medium at pH 7 containing 3 per cent cottonseed flour (Pharmamedia) and 2 per cent glucose. This seed medium was heavily seeded with spores of *S. filipinensis* from an agar slant of a similar composition. Preliminary studies indicated that the best nitrogen sources for growth and filipin synthesis were soy and cottonseed meals and flours.

Stability of filipin.—The antifungal factor in the fermented broth, or in solutions of crude preparations is heat labile. Fermented broths heated at 100° for 10 and 60 minutes lost 43 per cent and 79 per cent of their activity, respectively. A crude preparation



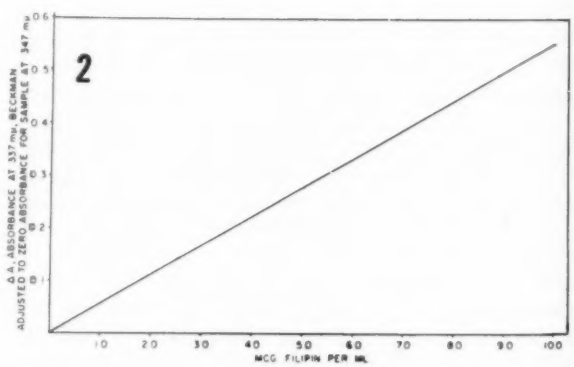


Fig. 1–2. Fig. 1. Ultraviolet absorption spectrum of crystalline filipin.—Fig. 2. Ultraviolet assay curve for filipin.

[4] V-8 juice, 350 ml; $CaCO_3$, 5 g; agar, 41 g; distilled water, 1000 ml. V-8 is the trade name of a mixture of vegetable juices manufactured by Campbell Soup Company.

[5] Vatsol is a detergent produced by American Cyanamid, Agricultural Chemical Division.

(500 units/mg in 25 per cent methanol) heated at 100° for 10 minutes lost 77 per cent of its activity. In 18 hr. at 23°, the fermented broth was stable at pH 7.9, lost 66 per cent of its activity at pH 2.2 and 48 per cent at pH 10.6. Under the same time and temperature conditions, the crude preparation lost 74 per cent of its activity at pH 2.5 and 81 per cent at pH 10.3. Filtration through Seitz or sintered glass filters removed only a little activity from a solution of the crude antibiotic. Ten per cent horse serum inactivated approximately 70 per cent of the antibiotic in a 20 per cent methanol solution.

EXTRACTION OF FILIPIN.—Filipin can be extracted from the culture filtrates and from the mycelia. The filtrate was extracted with 3 half-volumes of ether. The ether solution was evaporated to 1/10 volume, and the precipitate was collected and filtered. Evaporation to dryness destroyed some activity. The precipitates were washed 3 times with petroleum ether and dried under vacuum. Filipin is readily extracted from the mycelium with methanol or butanol. It was crystallized by trituration with chloroform and was recrystallized from methanol.

CHEMICAL PROPERTIES.—Filipin is soluble in methanol, ethanol, *n*-butanol, pyridine, dimethylformamide, and glacial acetic acid. It is nearly insoluble in water. To obtain aqueous solutions, the crude material was first dissolved in methanol or ethanol; the alcohol was then diluted with water and later removed under reduced pressure.

Filipin contains no nitrogen, sulfur, or halogen. It gives negative ninhydrin, biuret, Benedict, Tollens, and $FeCl_3$ tests. A deep blue color appears in concentrated HCl or concentrated H_2SO_4, but not in concentrated HNO_3. An elementary analysis of the crystalline material gave C = 62.95 per cent, H = 8.92 per cent.

The ultraviolet absorption spectrum in methanol or in 95 per cent ethanol solution showed peaks of maximum absorption at 322–323 mμ, 338 mμ, and 355–356 mμ; and minima at 328 mμ and 347 mμ (Fig. 1). The ultraviolet absorption spectrum of filipin places this new antibiotic in the polyene type group of antibiotics, yet it differs from other members of this series in the position of the 3 absorption peaks (3, 6).

The chromatographic behavior of crude filipin in various solvents is given in Table 1. R_f values were calculated from inhibition zones on bioautographs. These were made by placing the developed chromatograms on agar trays seeded with *P. oxalicum*.

TABLE 1.—*R_f values of filipin*[a]

Solvent system	R_f
H_2O saturated *n*-butanol	0.91
n-Butanol: acetic acid: H_2O (2:1:1)	0.95
n-Butanol: pyridine: H_2O (1:0.6:1)	0.96
3 Per cent NH_4Cl	0.00
50 Per cent hydrous acetone	0.72
n-Butanol saturated H_2O	0.00
Benzene: acetic acid: H_2O (2:2:1) (organic layer)	0.18

[a] On Whatman No. 1 filter paper and at a temperature of 24°C.

BIOLOGICAL PROPERTIES.—*Antimicrobial spectrum.*—Crystalline filipin was tested against a number of microorganisms by a dilution technique. The antibiotic was added to agar and the test organisms were streaked on the surface of the agar. The minimum inhibitory concentration in μg/ml is recorded for a number of organisms in Table 2. These data indicate that filipin is very active against fungi. Two yeasts, *Saccharomyces pastorianus* Hanson and *Zygosaccharomyces prioranus* Klöcker, also were inhibited at 5 μg/ml. Similar tests with bacteria showed no inhibitory action at 1000 μg per ml against the following organisms: *Micrococcus pyogenes* var. *aureus* (Rosenbach) Zopf, *Streptococcus pyogenes* Rosenbach, *Bacillus subtilis* Cohn emend. Prazmowski, *Escherichia coli* (Migula) Castell. & Chalmers, *Klebsiella pneumoniae* (Schroeter) Trev., *Salmonella schottmuelleri* (Winslow et al) Bergey et al, *Salmonella typhosa* (Zopf) White, *Shigella madampensis* (Castell.) Weldin, *Pseudomonas aeruginosa* (Schroe.) Mig., and *Proteus vulgaris* Hauser.

Fungicidal activity of filipin.—The fungicidal activity of filipin was determined by adding solutions of the antibiotic to heavy suspensions of *Candida albicans* or of spores of *P. oxalicum*. The mixtures were placed on a reciprocal shaker at 26°. Samples were taken at regular intervals and streaked on Emerson's-agar plates which were then incubated 24 hours (*C. albicans* at 30°, and *P. oxalicum* at 26°) to determine the viability of the cells or spores. Death of *C. albicans* cells occurred in 2 hours at a concentration of 50 μg/ml or in 24 hours at a concentration of 10 μg/ml. Higher concentrations or longer exposure had to be used to kill the spores of *P. oxalicum* (Table 3).

Antitrichomonal activity of filipin.—In CPLM (cysteine-peptone-liver-infusion-maltose-serum agar) medium, filipin killed *Trichomonas foetus* Riedmueller at a concentration of 50 μg per ml, markedly inhibited its multiplication at 25 μg per ml, and has some effect, more or less delayed, at concentrations as low at 1 μg per ml.[6]

Phytotoxicity and disease control.—Crude filipin had no phytotoxic effect upon the germination of pea and tomato seeds at concentrations of 100 μg/ml or less. Seeds were placed in methanol solutions of filipin. Thomas Laxton pea seeds were soaked for 1 hour and Blackhawk tomato seeds for 3 hours. The seeds were then placed in petri dishes containing 2 layers of filter paper saturated with nonsterile tap water. Twenty pea seeds per plate, replicated 10 times, and 50 tomato seeds per plate, replicated 4 times, were used in these trials. The seeds were incubated for 6 days at 23° and percentage germination was determined each day. Since the experiment was

[6] The writers wish to thank Dr. Norman Levine of the College of Veterinary Medicine for these data.

TABLE 2.—*Minimum inhibitory concentration of filipin against various microorganisms*

Test organism	μg/ml 48 hours	72 hours	96 hours
Saprophytes and plant pathogens[a]			
Aspergillus niger. v. Tiegh.		3.9	31.0
Colletotrichum lindemuthianum (Sacc. & Magn.) Bri & Cav.		1.9	3.9
Dendrophoma obscurans (Ell. & Ev.) H. W. Anderson		7.8	7.8
Diplodia sp.		31.0	1000.0
Endothia parasitica (Murr.) H. W. Anderson & P. J. Anderson		1.9	1.9
Fusarium oxysporum dianthi (Prill. & Del.) Snyd. & Hans.		7.8	15.0
Gnomonia fragariae Kleb.		0.95	0.95
Helminthosporium sativum (Pam.) King & Bakke		7.8	7.8
Penicillium digitatum Sacc.		3.9	7.8
Penicillium notatum Westling		31.0	62.0
Phoma betae Fr.		3.9	7.8
Phomopsis sp.		3.9	7.8
Rhizoctonia solani Kühn		500.0	500.0
Sclerotinia sclerotiorum (Lib.) d By		7.8	15.0
Thielaviopsis sp.		3.9	3.9
Human pathogenic fungi[b]			
Candida albicans (Robin) Berkhout	7.7		
Coccidioides immitis Rixford & Gilchrist	15.0		
Cryptococcus neoformans (Sanf.) Vuill.	0.95		
Geotrichum sp.	31.0		
Histoplasma capsulatum Darling	7.7		
Hormodendrum compactum Carrion	15.5		
Microsporum audouini Gruby	7.7		
Monosporium apiospermum Sacc.	7.7		
Nocardia asteroides (Epp.) Blanchard	31.0		
Phialophora verrucosa Thaxt.	15.5		
Sporotrichum Schenckii Matr.	7.7		
Trichophyton rubrum (Cast.) Sabour.	7.7		
Blastomyces dermatitidis Gilchrist & Stokes	31.0		

[a] Tested in glucose 3 per cent, yeast extract 0.7 per cent, KH_2PO_4 0.5 per cent, agar 2 per cent.

[b] Tested in glucose 1 per cent, peptone 0.5 per cent, yeast extract 0.1 per cent, agar 2 per cent, pH 6.8.

not made under sterile conditions, a number of naturally occurring fungi grew on the pea seeds and caused decay. Penicillia, Aspergilli, and Phycomycetes were the most common fungi on these seeds. The following are the percentages of fungus-rotted seeds in the various treatments: distilled water control, 12; 10 per cent methanol control, 24; and 25 to 100 μg per ml of filipin, 0.5 to 1.5. At lower concentrations of filipin the percentage of decayed seeds increased as the concentration was decreased.

TABLE 3.—*Growth of Candida albicans and Penicillium oxalicum after exposure to filipin*

Solvent and concentration of filipin in μg/ml	Growth[a] on Emerson's-agar plates *C. albicans* 2 hours	7 hours	24 hours	*P. oxalicum* 2 hours	7 hours	24 hours
Distilled water						
0	++	++	++	++	++	++
5% Methanol						
0	++	++	++	++	++	++
1	++	++	++	++	++	++
10	++	+	—	++	++	+
50	—	—	—	++	+	—
100	—	—	—	+	—	—

[a] ++ indicates good growth, + weak growth, and — no growth.

Filipin partially protects tomato from gray leaf spot, caused by *Stemphylium solani* G. F. Weber. Young tomato plants were sprayed with crude filipin at a concentration equivalent to 415 μg of pure filipin per ml of 25 per cent methanol. No phytotoxic effects were observed on sprayed noninoculated controls. Incidence of gray leaf spot on filipin-treated plants was 25 per cent less than on the controls. An alcoholic solution containing 50 μg/ml of filipin inhibited germination of spores of *S. solani* on glass slides.

COMPARISON OF FILIPIN WITH OTHER ANTIFUNGAL AGENTS PRODUCED BY ACTINOMYCETES.—That filipin is a new antibiotic was established by many criteria. Whenever possible, authentic samples of the other antibiotics were obtained to use for comparison. If these could not be obtained, the descriptions in the literature were used. Often even published data were not very definite or the antibiotics were still in the crude stage when they were reported. The most useful data for comparison have been ultraviolet absorption spectra, R_f values in different solvent systems, the inhibitory spectra, solubility data, and compositions of the compounds. Filipin differed from each of these other antibiotics in one or more of these properties, hence it appears to be a new antibiotic.

Recently, a new antibiotic, fungichromin, has been reported (5). Exploratory tests indicate that fungichromin is not identical with filipin. Studies of the differences between these 2 compounds as well as further details on the chemistry of filipin will be reported in a future publication.

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INHERITANCE OF RESISTANCE TO CUCUMBER MOSAIC VIRUS IN COWPEA[1]

J. B. Sinclair and J. C. Walker

SUMMARY

Certain strains of cucumber mosaic virus cause a local-lesion reaction in varieties of cowpea (*Vigna sinensis* Savi.) resistant to that virus and become systemic in susceptible varieties. Crosses were made between certain resistant varieties and 1 susceptible line derived from the resistant variety Black. The reactions of F_1, F_2, and F_3 progenies proved that resistance is dominant and governed by a single gene pair. Approach-graft experiments indicated that the virus did not move from susceptible into resistant plants sufficiently to become established and that the resistant plants were not systemically hypersensitive.

The cowpea (*Vigna sinensis* Savi.) is commonly used as an assay host for cucumber mosaic virus (CMV). It was shown, however, by Whipple and Walker (7) and by Fulton (1) that some strains of the virus isolated from legumes become systemic in cowpea. Thus, a cowpea variety may be designated as resistant to strains that produce local lesions on it and susceptible to the strains of the virus that become systemic. In working with strains of CMV on cowpea, it was noticed by Sill and Walker (5) that an occasional plant in the variety Black became systemically infected by strains that normally produce local lesions on that variety. When seed was grown from a number of such plants, all progenies uniformly gave a systemic reaction to strains of the virus that produced local lesions on the nonselected Black. The 2 lines were indistinguishable by plant characters. The susceptible line of Black is designated herein as Bsy and the typical resistant variety of Black as Bll.

The present study was planned to determine 1) the inheritance of the character responsible for the systemic reaction of the susceptible line in relation to that governing the local-lesion reaction of resistant varieties, and 2) whether a systemic necrotic reaction of resistant plants could be produced by introducing a necrotic strain of the virus from susceptible plants by approach grafts.

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Procedure.—Parental material for the study consisted of the susceptible line of Black (Bsy), the typical resistant Black variety (Bll), the resistant variety Black Eye (BE), and the resistant variety Dixie Queen (DQ). The variegated seed color of BE and DQ was advantageous because it distinguished them from the solid black color of Bsy. Any chance selfs of the maternal parent could then be determined by the lack of segregation of seed color of F_2 seed. All crosses were made in the greenhouse. It was found that the cowpea was selfed readily before the flowers opened and that any crosses would have to be made 24 to 36 hours before blossoming. Emasculation at that time was followed by hand pollination. Care was taken to cause as little damage as possible to the petals, which were used to protect the pistil. It was found also that only a small portion of the stigma near the tip was receptive. This area was readily identified by the green color, which was prominent in unopened flowers. A similar stigma receptivity has been reported in pea (*Pisum sativum* L.) by Warnock et al (6). F_1 plants of the crosses were grown to maturity in the greenhouse. The parent lines and F_2 seed were started in plant bands and transplanted to field plots for increase.

Tests for resistance or susceptibility were conducted in the greenhouse. To facilitate uniform germination, seeds were treated with concentrated sulfuric acid for 15 minutes and placed on moist filter paper in petri dishes. After germination they were planted in Vermiculite in shallow baked clay trays to which a com-

TABLE 1.—*Segregation of F_2 populations from 10 crosses into resistant and susceptible classes*

Cross	Number of plants in class indicated		χ^2	
	Resistant	Susceptible	3:1	P range
Bsy-6 × Bll-2	118	42	0.08	0.80-0.70
Bsy-7 × Bll-8	51	19	0.08	0.80-0.70
Bsy-8 × BE-1	749	260	0.28	0.70-0.50
BE-2 × Bsy-1	606	198	0.04	0.90-0.80
BE-3 × Bsy-2	211	55	2.43	0.20-0.10
BE-4 × Bsy-3	112	45	0.94	0.50-0.30
BE-5 × Bsy-5	167	46	1.14	0.30-0.20
DQ-1 × Bsy-2	391	129	0.01	0.95-0.90
DQ-2 × Bsy-3	217	83	1.00	0.50-0.30
DQ-3 × Bsy-4	70	34	2.89	0.10-0.05
Total	2692	911	0.14	0.80-0.70

plete nutrient solution was applied when needed. Just before unifoliate leaves were fully expanded, the plants were covered for 24 hours whereupon they were inoculated according to the method of Sill and Walker (4). The strain of CMV used was one that induced only local lesions on cowpea varieties herein designated as resistant. It was used originally by Kuntz and Walker (3) and later by Sill and Walker (4), who designated it as strain Y.

RESULTS.—All F_1 plants resulting from crosses between resistant and susceptible parents were resistant and responded to inoculation by formation of local lesions only. None of the F_2 progenies from 10 crosses deviated significantly from the ratio of 3 resistant to 1 susceptible (Table 1).

F_3 progenies were grown from 2 crosses. The classification of F_3 families is indicated by the data in Table 2. These data are a close fit to the expected ratio of 1 homozygous resistant to 2 heterozygous to 1 homozygous susceptible.

The close fit of actual to the expected for a 3:1 segregation of plants in the F_2 and of actual to expected distribution for a 1:2:1 ratio in the F_3 proves that resistance and susceptibility are controlled by a single gene pair.

Grogan and Walker (2) found that bean varieties derived from Corbett Refugee that were resistant to common bean mosaic virus reacted with a systemic necrosis when this virus was introduced from a susceptible variety through approach grafts. It was

TABLE 2.—*Distribution of F_3 families from 2 crosses between resistant and susceptible parents*

Cross	Number of progenies in class indicated[a]			χ^2	
	R	3R:1S	S	1:2:1[b]	P range
Bsy-6 × Bll-2	4	11	5	0.30	0.70-0.50
Bsy-7 × Bll-8	3	10	3	1.00	0.50-0.30
Total	7	21	8	1.06	0.50-0.30

[a] R indicates resistant and S, susceptible.
[b] Homozygous resistant to heterozygous to homozygous susceptible.

thought that a similar situation might obtain between resistant and susceptible cowpeas. If this were the case, the resistant cowpea would react with a systemic necrosis if a strain of CMV that produces local lesions in resistant varieties were introduced from the susceptible cowpea plant through an approach graft. Twenty-five approach grafts were made between resistant and susceptible plants ranging in age from the 2- to the 6-trifoliate-leaf stage. Seeds of the 2 lines were planted in soil in the same pot and approach grafts were made between the plants. After the grafts were complete the susceptible plants were inoculated with strain Y of CMV. In some cases the resistant plants were shaded for two 6-day periods and one 8-day period, with each period separated by 8 days without shading. It was thought that the reduction in photosynthesis in the resistant plant by shading would encourage translocation from the susceptible plant to the resistant plant and therefore increase the movement of the virus into the resistant plant. The resistant plant in most of the grafts was severed from its root after the graft was complete so that it became completely dependent upon the susceptible plant for water and minerals. In no case did the resistant plants show any sign of necrosis, and none died during the experiments. These results indicated that the virus did not move from the susceptible into resistant plants sufficiently to become established and that the resistant plants were not systemically hypersensitive.

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THE INFLUENCE OF INFECTION BY RING SPOT VIRUS UPON THE GROWTH OF ONE-YEAR-OLD MONTMORENCY NURSERY TREES[1]

D. F. Millikan[2]

SUMMARY

Comparison in total growth between nursery-run noncertified Montmorency and the same clone propagated from ring-spot-free certified sources indicated that the latter were significantly more vigorous. Comparisons between Montmorency trees propagated from ring-spot-free and artificially inoculated sources indicated that the growth-depressing factor could be the ring spot virus. Some strains were so mild, however, that this inhibition was detected only during the middle part of the growing season and was not significant near the end of the growing season. The greatest difference in growth appeared to be due to the greater amount of branching of the ring-spot-free controls. The average heights of the ring-spot-free and infected trees were approximately the same. Most of this growth difference occurred during the June-July period and was completely absent during the first 6 weeks.

Ring spot of stone fruits, a virus disease, was described by Cochran and Hutchins (4) in 1941. A similar virus disease, necrotic ring spot, was reported independently by Chamberlain et al (1) and Hildebrand et al (5) the following year. Since that time the virus or virus complex causing ring spot has been recognized by Cochran (3) as a common contaminant in other stone fruit virus cultures. The economic aspects of this disease have been evaluated by several workers. Lewis (6) cites reductions in the yields of sour cherry up to 40 per cent during the year of initial infection, whereas Moore (10) noted similar reduction in yields only on trees infected also with sour cherry yellows virus. It is the belief of the workers at the Wisconsin Experiment Station (13) that adverse effects, if any, due to necrotic ring spot are limited to the year when acute symptoms occur.

Infection by the ring spot virus has been associated with a decline in the vigor of stone fruit trees in Utah (2), and the group of latent viruses was observed by Milbrath to cause a marked reduction in the growth of sweet cherry nursery trees. Two preliminary papers (8, 9) reported results similar to Milbrath's observations but did not eliminate the sour cherry yellows virus as the causative entity in the observed growth inhibition. Growth inhibition due to infection with ring spot virus has been reported also by Parker, Brase, and Klos (11).

The results reported in this study include 2 years' observations. During the 1951 season, comparisons were made between nursery-run noncertified stocks and stocks free of ring spot. Inasmuch as this study indicated that the nursery-run stocks were appreciably less vigorous than stocks propagated from indexed sources, the study in 1953 involved comparisons between stocks propagated from sources free of ring spot and from artificially inoculated sources.

MATERIALS AND METHODS.—During the 1950 propagating season, budsticks were collected randomly from nursery-run Montmorency trees, and buds from these were budded into seedlings of *Prunus mahaleb* grown from seed produced in the western states. Similarly, buds were collected from indexed Montmorency sources free from ring spot and were budded into seedlings in an adjoining row. Measurements were made at intervals during the 1951 season on the average height and average total growth. Average total growth was determined by summing all measurements of shoot growth that occurred on the individual trees. Inasmuch as the average height did not prove to be a reliable index for comparison purposes, only the data on total growth were analyzed.

Material was not available for the study during the 1952 season. In 1953, stocks propagated from 4 infected sources, which had been artificially inoculated with different strains[3] of ring spot virus known to be free from the cherry yellows virus, were compared with certified material. Bud sources of each treatment were single trees 1 generation removed from a parent tree. Measurements were made at approximately 2-week intervals and the growth for any 1 period was the difference between the total growth occurring during the previous period and that found for the date under consideration. Inasmuch as the 1953 season was exceptionally hot and dry, growth after the middle of August had ceased and observations thereafter were discontinued. The data were subjected to the *t* test described by Snedecor (12).

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[2] Plant Pathologist, Department of Horticulture, University of Missouri, Columbia. Most of this work was completed while the author was a member of the Botany Department. Appreciation is extended to Drs. T. W. Bretz and A. B. Stephenson of the Forestry and Poultry Departments, respectively. Both men read the manuscript and offered helpful suggestions.

[3] Strains of the ring spot virus used in the study and original source are as follows: Goldman 2-1 and Barnard 1-12 from J. D. Moore of the Wisconsin Agricultural Experiment Station; E-253 from D. Cation of the Michigan Agricultural Experiment Station; and tatter leaf from G. H. Berkeley of the Dominion Laboratory of Plant Pathology, St. Catharines, Ontario.

TABLE 1.—*Difference in growth of 1-year-old ring-spot-free Montmorency cherry trees and that of comparable trees infected with different strains of ring spot virus*

Period	Total shoot growth of ring-spot-free trees[a]	Mean difference between total shoot growth of virus-free and infected sources[b]			
		Tatter	Goldman 2-1	Barnard 1-12	E-253
	in.	in.	in.	in.	in.
Prior to June 3	59.6	−7.6	−13.5	−2.6	−5.5
June 4 to June 16	37.6	−14.9	−26.5**	−18.6**	−18.2*
June 17 to July 3	41.2	−19.2**	−15.1**	−17.0**	−11.6
July 4 to July 20	38.6	−24.5**	−18.3**	−19.7**	3.9
July 21 to Aug. 3	29.1	4.0	−5.0	−7.8	5.1
Aug. 4 to Aug. 16	17.9	1.7	−2.2	−5.0	−9.5
Total	224	−60.5**	−80.9**	−70.7**	−35.8

[a] Mean growth per tree, based on 16 trees.

[b] The symbol * indicates significance at the 5 per cent level; ** indicates significance at the 1 per cent level. A minus sign indicates that the mean for 16 measured infected trees was less than that for the ring-spot-free trees.

RESULTS WITH NURSERY-RUN MATERIAL. — Observations on the total growth of Montmorency trees indicated an average of 186 and 245 in. for the material propagated from nursery-run and ring-spot-free sources, respectively. a difference of about 30 per cent. This difference is significant at the 1 per cent level, indicating that noncertified cherry stocks were less vigorous than those propagated from ring-spot-free sources.

The correlation coefficient of average growth and average height indicated r values of 0.15 and 0.49 for the ring-spot-free and nursery-run trees, respectively. This indicated that for the 1951 season, only about 2 per cent of the variation in total growth of the ring-spot-free trees was related to average height. In the case of the nursery-run material, about 25 per cent of the variation appeared to be related to average height. The low correlation coefficient between height and total growth can be explained by the greater number of branches in the disease-free stocks than in the nursery-run material.

RESULTS WITH RING-SPOT-INFECTED MATERIAL.—Data on the growth of ring-spot-affected and ring-spot-free 1-year-old Montmorency trees are listed in Table 1. Analysis of the data indicated that certain strains of ring spot virus, including Goldman 2-1, tatter leaf, and Barnard 1-12, caused a significant reduction in growth. This reduction, however, was not apparent during the period of growth up to the first part of June and was absent during the last part of the growing season. This is in agreement with other observations (8, 9) made previously. This growth was cumulative and significant near the end of the growing season and thus in agreement with Milbrath's observations (7). On the other hand, not all strains of the ring spot virus react the same, and this is demonstrated by the observations on the E-253 infected trees. Only in the second period under observation was there any significant decrease in growth detected, and this decrease was not sufficient to cause any significant difference at the end of the growing season.

The correlation coefficient of average total growth and average height indicate r values of 0.61 and 0.59 for the disease-free and infected trees, respectively.

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HOST NUTRITION IN RELATION TO CONCENTRATION OF POTATO VIRUS X AND TOBACCO RING SPOT VIRUS [1]

Katie Helms and Glenn S. Pound

SUMMARY

Nicotiana glutinosa, *N. tabacum*, and *Cucumis sativus* were grown in sand cultures watered with nutrient solutions containing different concentrations of nitrogen, of phosphorus, and of a balanced nutrient solution. Plants of the *Nicotiana* species were inoculated with potato virus X and the cucumber plants with tobacco ring spot virus. Effects of host nutrition on concentration of the viruses in the hosts were determined in assays made approximately 7 and 14 days after inoculation. Optical-density readings on partially purified virus extracts from plant sap were made with both viruses, and local-lesion assays also were made with virus X.

Of the 3 nutrient series used, nitrogen produced the most marked effects on host growth, symptom expression, and virus concentration of both viruses. Mild symptoms and low virus concentrations developed at the lowest nitrogen level, and symptom severity and virus concentration increased with increasing concentrations of nitrogen. The maximum nitrogen concentration used was 1050 ppm. At this level, excess nitrogen caused stunting of the *Nicotiana* species and death of cucumber plants.

Low phosphorus levels were associated with mild symptoms and stunting of all hosts. There was little difference in host growth or symptoms at the higher phosphorus levels. Concentration of both viruses generally increased with increased phosphorus. In some experiments the highest concentration was in plants grown at 547 ppm, the highest level, and in others it was in plants grown at 237 ppm.

With balanced nutrient concentrations, maximum growth of plants of *N. tabacum* and of *N. glutinosa* infected with virus X was at 0.5H or 1H (the basal level). Maximum concentration of virus X was usually in plants grown at 1H, although in some experiments it was in those grown at 2H. Maximum growth of cucumber was at 0.5H, and growth decreased with increasing nutrient concentration. The data suggested that variations in osmotic concentrations did not strongly affect multiplication of virus X or tobacco ring spot virus, except at the 3H level which consistently caused reduction of virus concentration.

Methods used for estimation of virus were considered adequate where differences among treatments were large, but were not of sufficient accuracy where differences among treatments were small.

The study of effects of host nutrition on virus multiplication is a relatively new field, although there are many reports of experiments on effects of host nutrition on susceptibility of plants to virus infection. Results of early experiments on multiplication of tobacco mosaic virus in relation to nutrition were inconsistent. This was perhaps due to variations in experimental methods. In a recent series of papers, Pound and associates (1, 6, 8) have shown, in experiments similar to those to be described, that host nutrition markedly affects virus concentration of turnip mosaic virus in *Nicotiana glutinosa* L. and in *N. bigelovii* var. *multivalvis* (Lindley) East, of cucumber mosaic virus in spinach, and of tobacco mosaic virus in *N. tabacum* L.

This paper gives data on the effects of 4 concentrations of nitrogen, of phosphorus, and of a balanced nutrient solution on multiplication of potato virus X and tobacco ring spot virus.

MATERIALS AND METHODS.—Multiplication of the clear ring strain of virus X described by Ladeburg, Larson, and Walker (3) was studied in *Nicotiana tabacum* var. Havana 38 and in *N. glutinosa*, and that of the common green strain of tobacco ring spot virus was studied in *Cucumis sativus* L. var. National Pickling. These 2 viruses were maintained in the greenhouse in *N. glutinosa* and cucumber, respectively, as well as in desiccated tissue.

Tobacco seeds were sown in white quartz sand and watered with a balanced nutrient solution. Young seedlings were transplanted into trays of sand, and selected uniform seedlings were later planted into 9-in. glazed earthenware crocks filled with white silica sand and fitted with automatic siphons as described by Pryor (7). Cucumber seeds were sown in vermiculite, and when the cotyledons were expanded, seedlings were transplanted into crocks of sand as described for tobacco. There were 5–6 seedlings per crock in virus X studies, and 8–10 in ring spot virus studies. The crocks were kept well watered with the prescribed nutrient solutions, which were modifications of a solution formulated by Hoagland and Snyder (2). These modified solutions were identical with those used by Weathers and Pound (8) and first described by Cheo, Pound, and Weathers (1), with the exception that in the nitrogen series for some of the tobacco ring spot studies, concentrations of 5, 21, 210, and 630 ppm nitrogen were used instead of 21, 210, 630, and 1050 ppm. There were 2 or 4 replicates of each of 4 nutritional levels in each experiment.

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When differential growth responses to nutrition were apparent, plants in half the crocks of each treatment were inoculated with a sap solution of the virus. Plants were first dusted with 600-mesh carborundum, and inoculum was applied evenly with a cheesecloth pad to 2–3 leaves of each tobacco plant and to the cotyledons or first true leaves of cucumber plants. All plants in a given experiment received identical inoculations. Harvesting of 1 crock of inoculated plants and 1 crock of noninoculated plants in each treatment was done at approximately 7- and 14-day intervals after inoculation for *Nicotiana* species and at approximately 10- and 18-day intervals for cucumber. Plants were cut at sand level and weighed. The leaf and stem tissues were frozen for a minimum period of 24 hours. In some experiments, inoculated leaves were harvested and weighed separately. When the quantity of tissue was small, sap extracts for virus concentration measurements were obtained by grinding the frozen tissue with a mortar and pestle; when the amount of tissue was larger, it was passed through a food chopper or juice extractor. The sap was squeezed through 2 layers of cheescloth and liquid aliquots were partially purified by ultracentrifugation as described by Pound and Helms (5).

In experiments with virus X, relative concentrations of virus nucleoprotein were determined by optical-density readings, and infectivity measurements were made by a local-lesion technique using *N. glutinosa* held at 16°C as the assay host. Details of these methods used for virus X were described in a previous paper (5). When extracts from cucumber plants infected with tobacco ring spot virus were prepared by the method just described, the ultraviolet-absorption values of virus fractions taken at 5-mμ intervals in the range of 230–300 mμ, minus the corresponding fractions from noninfected plants subjected to the same treatment, were characteristic of purified tobacco ring spot virus and reached a maximum at 260 mμ (4). Relative concentrations of virus nucleoprotein in cucumber plant extracts, like those of virus X, therefore were obtained directly on a Beckman DU spectrophotometer by using the fractions from healthy plants as blanks for optical-density readings of the virus extracts. It should be pointed out that these optical-density assays were measurements of total extractable nucleoprotein, whereas the local-lesion assays measured only virus infectivity. Presumably, the former measured virus nucleoprotein.

EXPERIMENTAL RESULTS.—*Virus X.*—1) *Nitrogen.*—*N. tabacum* was grown in nutrient solutions containing 21, 210, 630, and 1050 ppm nitrogen. Experiments were made throughout the year, and variations in growth were considerable. The mean fresh-weight yields per plant, averaged over 13 harvests for several experiments for nitrogen levels of 21, 210, 630, and 1050 ppm, were 5.1, 21.3, 21.7, and 19.3 g, respectively, for inoculated plants and 7.0, 22.8, 24.2, and 19.6 g, respectively, for noninoculated plants. The mean maximum fresh weight for both inoculated and healthy plants was therefore at 630 ppm, although occasionally maxima were obtained at 210 and 1050 ppm. Mean growth of inoculated plants at 210 ppm almost equalled that at 630 ppm.

One week after inoculation, plants grown at 21 ppm were chlorotic; they showed no systemic symptoms, but sometimes there were ring spot lesions on inoculated leaves. At 210 ppm, plants showed a well-developed ring spot pattern on inoculated leaves and a mild to severe systemic mottle. Symptoms at 630 ppm closely resembled those at 210 ppm. At 1050 ppm, plants were dark green and stunted; they showed a distinct ring spot pattern on inoculated leaves but only a slight systemic mottle. Two weeks after inoculation, the systemic mottle on plants grown at 210 and 630 ppm was more severe than that on plants grown at 21 or 1050 ppm.

Data for *N. tabacum* in Table 1 are representative of those obtained in 5 experiments. Virus concentration, as measured by optical density and by local-lesion counts in tests with sap of either whole plants or inoculated leaves, increased with increasing concentration of nitrogen, at both 7 and 14 days after inoculation. In only 1 of 9 spectrophotometric assays did virus concentration data resemble growth data and show a maximum in plants grown at 630 ppm rather than in those grown at 1050 ppm. Local-lesion assays were more variable but showed essentially the same concentration gradient. In 2 of 9 assays, the number of lesions from extracts of plants grown at 630 ppm was greater than that from extracts grown at 1050 ppm but not significantly so. In all 9 assays, the number of lesions from plants grown at 21 ppm was significantly less than at least 1 of the higher concentrations; in 5 assays it was significantly less than that from plants grown at 210, 630, or 1050 ppm. Differences between lesion counts at 210 and 610

TABLE 1.—*Optical-density and local-lesion assays of virus X in extracts from N. glutinosa and N. tabacum grown at 4 levels of nitrogen*

Days after inoculation	Nitrogen levels (ppm)	*N. tabacum*		*N. glutinosa*
		Optical density	Average no. local lesions per half leaf[a]	Optical density
7	21	0.230	19.7	0.073
	210	0.618	69.4	0.108
	610	0.750	77.7	0.128
	1050	1.280	142.6	0.136
14	21	0.150	97.4	0.167
	210	0.498	133.8	0.218
	610	0.560	133.3	0.311
	1050	0.820	160.0	1.00
7[b]	21	0.207	12.3	
	210	0.670	15.6	
	610	0.750	22.0	
	1050	1.300	30.3	

[a] Based on 9 half leaves.
[b] Assay made of inoculated leaves. Other assays were from systemically infected leaves.

ppm were significant in only 2 assays. The highest virus concentration was thus recorded at the highest nitrogen level, which also caused stunting of plants.

N. glutinosa was grown at the same nitrogen levels as *N. tabacum*. One week after inoculation, plants at all 4 nitrogen levels showed well-developed concentric or necrotic ring spots. Lesions on plants grown at higher concentrations tended to coalesce and were more numerous than those on plants grown at 21 ppm. Except at this low nutrient level, plants showed a conspicuous yellow-green systemic mottle (Fig. 1). There was little or no difference in symptoms of plants at the 3 higher levels. Plants grown at 21 ppm were chlorotic and those grown at 1050 ppm were dark green as compared with those grown at 210 and 630 ppm. Averaged over 11 harvests, the mean fresh weights (per plant) in treatments of 21, 210, 630 and 1050 ppm were 3.6, 7.7, 8.1, and 6.1 g, respectively, for inoculated plants and 4.1, 9.3, 9.4, and 6.8 g, respectively, for noninoculated plants.

Data for *N. glutinosa* in Table 1 are representative of those obtained in 6 of 9 spectrophotometric assays and resemble data for *N. tabacum*. Lowest optical-density readings were in extracts of plants grown at 21 ppm, and values increased with increasing concentration of nitrogen to a maximum in plants grown at 1050 ppm. The same pattern was evident at 7 and 14 days after inoculation. In 2 assays of 1 experiment, the maximum was unaccountably at the 610-ppm treatment in the first assay and at the 210-ppm treatment in the second; in the second assay of another experiment, the maximum was at the 610-ppm treatment. Differences in these experiments, however, were small.

Assays by the local-lesion technique showed an increase in virus concentration as the amount of nitrogen supplied the source plants was increased. Differences in the infectivities of extracts from plants grown at 21 ppm and those of plants grown at 1050 ppm were significant, but other differences were too small to be significant.

The data showed that for both *N. tabacum* and *N. glutinosa*, concentration of virus X increased with increase of nitrogen and that differences in concentrations in plants grown at 210, 630, and 1050 ppm were usually less marked in *N. glutinosa* than in *N. tabacum*.

2) *Phosphorus.*—Nutrient solutions contained 3, 93, 237, and 547 ppm of phosphorus. In experiments with *N. tabacum*, maximum fresh weight was usually at 237 ppm, but in some experiments it was at 93 or 547 ppm. The mean fresh weights (per plant), averaged over 9 harvests for *N. tabacum* plants grown at 3, 93, 237, and 547 ppm, were 7.3, 11.8, 13.4, and 13.2 g, respectively, for inoculated plants and 8.5, 22.6, 22.8, and 22.2 g, respectively, for noninoculated plants. Symptoms of plants grown at 3 ppm were mild and slow to appear. Local lesions consisted of concentric ring spots and necrotic specks, and systemic symptoms consisted of a green mottle. Symptoms of plants grown at the 3 highest phosphorus levels were more severe than those of plants at the lowest level. Two weeks after inoculation, symptoms on all groups were indistinguishable from each other.

Optical-density data showed that the lowest virus nucleoprotein concentration was always in plants grown at 3 ppm. In 5 assays, virus concentration increased consistently with increase of phosphorus to reach a maximum at the highest phosphorus level, and in 4 assays a maximum was reached at 237 ppm followed by a decrease at 547 ppm. There was no consistent difference between assays made 7 and 14 days after inoculation. Data from a representative experiment are shown in Table 2. Local-lesion data followed the same pattern as optical-density data; extracts from plants grown at 3 ppm always produced fewer lesions than those from plants grown at higher levels.

Experiments with *N. glutinosa*, as did those with *N. tabacum*, showed that differences in growth at the higher phosphorus levels were small. The mean fresh weights (per plant), averaged over 9 harvests for levels of 3, 93, 237, and 547 ppm, were 4.4, 7.5, 8.5, and 7.3 g, respectively, for inoculated plants and 5.6, 9.5, 10.7, and 9.7 g, respectively, for noninoculated plants. Plants grown at 3 ppm showed fewer local lesions and developed a systemic mottle more slowly than did plants grown at higher levels. Two weeks after inoculation, symptoms of plants grown at the 3 higher levels were indistinguishable from each other.

Optical-density data showed the smallest virus nucleoprotein concentration to be in plants grown at 3 ppm and the highest to be in plants grown at either 237 or 547 ppm (Table 2). The maximum was at the 237-ppm treatment in 3 assays and at the 547-ppm treatment in 3 others. This variation, as was that in tests with *N. tabacum*, was not associated with length of time following inoculation. Local-lesion data showed only small differences.

Thus in the experiments with *N. tabacum* and with *N. glutinosa*, the phosphorus concentration that was optimum for virus multiplication was in the range of 237 and 547 ppm. Neither maximum virus multiplication nor maximum growth was consistently associated with a single phosphorus level, although the lowest

TABLE 2.—*Optical-density assays of virus X in extracts of N. glutinosa and N. tabacum grown at 4 levels of phosphorus*

Days after inoculation	Phosphorus levels (ppm)	Optical density	
		N. tabacum	*N. glutinosa*
7	3	0.000	0.055
	93	0.205	0.091
	237	0.406	0.105
	547	0.495	0.218
14	3	0.025	0.282
	93	0.057	0.458
	237	0.186	0.515
	547	0.000[a]	0.295

[a] In this assay the optical-density value of the extract from control plants was equal to that of the extract from infected plants.

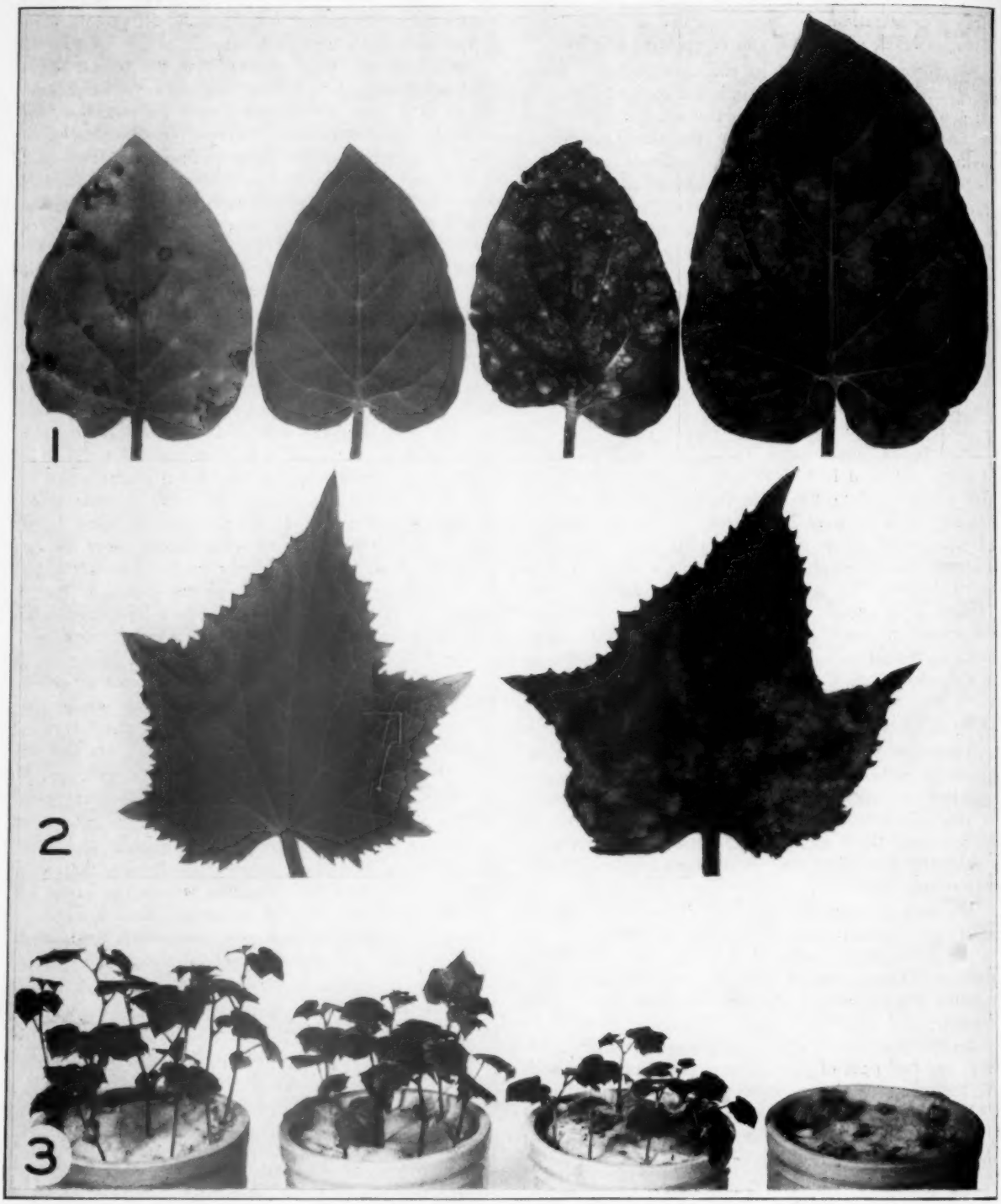

FIG. 1–3.—Fig. 1. Leaves from plants of *N. glutinosa* infected by virus X, 8 days after inoculation. The 2 leaves on the left are from plants grown at 21 ppm nitrogen and the 2 leaves on the right are from plants grown at 1050 ppm nitrogen. In each pair, the inoculated leaf is on the left, and a young systemically infected leaf is on the right.—Fig. 2. Young leaves of cucumber plants 12 days after inoculation of cotyledons with tobacco ring spot virus. Leaves are from plants grown at 5 ppm nitrogen (left) and 630 ppm nitrogen (right).—Fig. 3. Effects of 4 concentrations of a balanced nutrient solution on growth of cucumber plants 8 days after inoculation with tobacco ring spot virus. Nutrient concentrations were 0.5H, 1H, 2H, and 3H (left to right).

virus concentration was consistently associated with the lowest phosphorus level.

3) *Concentrations of balanced nutrient solution.*—*N. tabacum* was grown at 4 concentrations of a balanced nutrient solution. The basal solution was designated as 1H, and concentrations of solutions used were 0.5H, 1H, 2H, and 3H. At the 1H level, the concentrations of N, P, and K were 210, 31, and 235 ppm, respectively. Equal quantities of a trace-element solution were added to each treatment.

Four experiments were made with *N. tabacum*. Maximum growth at 3 harvests of inoculated plants and 2 harvests of noninoculated plants was at 0.5H, and at 1 harvest of healthy plants it was at 2H. At all other harvests it was at 1H. The mean fresh weights (per plant), averaged over 8 harvests for 0.5H, 1H, 2H, and 3H, were 11.3, 13.5, 10.4, and 5.2 g, respectively, for inoculated plants, and 13.4, 17.4, 11.0, and 7.6 g, respectively, for noninoculated plants.

One week after inoculation, plants in all treatments showed well-defined ring spots on inoculated leaves, and all plants except those grown at the 0.5H level showed a systemic mottle. Plants grown at 0.5H were a lighter green than those grown at 1H, whereas plants grown at 3H were a darker green and tended to wilt during the middle of the day. Two weeks after inoculation, plants grown at 0.5H had developed a systemic mottle. The mottle on plants grown at 1H was more severe than on those grown at 0.5H and was similar to that at 2H. Symptoms were less severe in plants at the 3H level.

In 4 of 7 assays, maximum virus concentration was in plants grown at 1H; virus concentration decreased as nutrient concentration was increased above 1H. Thus, virus concentration was correlated with symptom expression and followed the mean-growth pattern. In 1 assay, plants of all treatments contained approximately the same amount of virus, and in 2 assays the greatest virus concentration was at 2H.

Similar experiments were made with *N. glutinosa*. In 2 harvests of inoculated plants and in 2 harvests of noninoculated plants, maximum fresh weight was at the 0.5H level, but at all other harvests it was at the 1H level. The mean fresh weights (per plant), averaged over 9 harvests for 0.5H, 1H, 2H, and 3H, were 5.4, 5.1, 3.4, and 2.7 g, respectively, for inoculated plants and 6.1, 6.6, 5.0, and 3.7 g, respectively, for noninoculated plants. One week after inoculation, plants grown at 0.5H showed pronounced concentric ring spots on inoculated leaves and a systemic mottle with slight necrosis on younger leaves. Local lesions on plants grown at 1H and 2H were slightly less severe than those on plants grown at 0.5H, but systemic symptoms were similar on all 3 groups. Symptoms on 3H plants resembled those on 1H and 2H plants in some experiments, whereas in others they were milder. Two weeks after inoculation, systemic mottles and ring spot patterns were well developed in all treatments, but those on plants at 3H tended to be the least severe.

As in *N. tabacum*, multiplication of virus X in *N. glutinosa*, as measured by optical density, was not greatly affected by the osmotic concentrations used. In both assays of each of 2 experiments and in 1 assay of another experiment, maximum virus concentration was in plants grown at 1H, and virus concentration decreased at 2H and 3H. In 3 additional assays, there was little difference in virus concentration at the 3 higher nutrient levels. Data in 5 of 6 local-lesion assays followed the same pattern as the optical-density readings, reaching a maximum at 1H and decreasing at the higher levels. Differences in numbers of lesions among treatments, however, were small. Data obtained with *N. glutinosa* thus resembled generally those obtained with *N. tabacum*.

Tobacco ring spot virus.—1) *Nitrogen.*—In 4 experiments, effects of different concentrations of nitrogen on growth of cucumber were pronounced. As in virus X studies, experiments were first made at concentrations of 21, 210, 610, and 1050 ppm, but since plants were killed at 1050 ppm, levels of 5, 21, 210, and 610 ppm were used in 2 additional experiments. Maximum growth was at either 21 or 210 ppm, and plants at 5 ppm and 610 ppm were consistently stunted. Those at 5 ppm were chlorotic, whereas those at 610 ppm were dark green. The mean fresh weights (per plant) at 5, 21, 210, and 610 ppm were 1.6, 2.4, 2.8, and 2.5 g, respectively, for inoculated plants and 2.1, 3.6, 4.5, and 4.0 g, respectively, for noninoculated plants.

Symptoms were not always evident 7 days after inoculation but were after 10 days. At that time, inoculated leaves or cotyledons of plants grown at 5 ppm showed yellow local lesions, and plants at higher levels had developed a pronounced yellow-green systemic mottle. Young leaves of plants grown at 5 and 630 ppm nitrogen and photographed 12 days after inoculation are shown in Figure 2. Twenty days after inoculation, mild systemic symptoms were evident on plants grown at 5 ppm. Systemic symptoms on plants grown at the 3 higher levels were indistinguishable from each other and consisted of a yellow-green rugose mottle that caused slight distortion of young leaves.

Assays of sap samples of plants grown in both series of nitrogen concentrations consistently showed maximum virus nucleoprotein concentration in those grown at the highest nitrogen level and minimum concentration in those grown at the lowest. Representative data shown in Table 3 indicate that virus nucleoprotein concentration increases with increasing concentrations of nitrogen and reaches a maximum at a nitrogen level that causes stunting.

2) *Phosphorus.*—Six experiments, including 12 harvests and assays, were made in which phosphorus concentrations of 3, 93, 237, and 547 ppm were used. Minimum growth was in plants grown at 3 ppm, whereas maximum growth was either at 93 or 237 ppm, except in 2 harvests when it was at 547 ppm. The mean fresh weights (per plant), averaged over all harvests at levels of 3, 93, 237, and 547 ppm, were

3.3, 4.5, 4.8, and 3.8 g, respectively, for inoculated plants and 5.1, 6.3, 7.5, and 6.3 g, respectively, for noninoculated plants.

Symptoms 1 week after inoculation consisted of a mild systemic mottle on plants grown at 3 ppm and of a more severe mottle on plants grown at higher concentrations. Two weeks after inoculation, plants in all treatments showed a severe yellow-green mottle with distortion of young leaves. In some experiments, symptoms of plants grown at 237 ppm were the most severe, but in others symptoms at the 3 higher levels could not be distinguished from each other. Plants grown at 547 ppm were dark green, whereas plants grown at 3 ppm tended to be chlorotic.

Seven days after inoculation, the pattern of virus nucleoprotein concentration was irregular. At approximately 14 days after inoculation, maximum concentration was usually at the 237-ppm treatment, but occasionally it was at the 547-ppm treatment. Table 4 gives optical-density data from an experiment in which the maximum virus concentration was in plants grown at 547 ppm in the first assay and in those grown at 237 ppm in the second. Data of the second assay are representative of the mean data obtained in all 6 experiments. At approximately 14 days after inoculation, virus concentration showed a correlation with mean growth of the host.

3) *Concentration of a balanced nutrient solution.*— Four experiments were made, using the same nutrient concentrations used in corresponding studies on virus X. Growth was always at a maximum at 0.5H and decreased with increasing nutrient concentration (Fig. 3). The mean fresh weights (per plant), averaged over 8 harvests for 0.5H, 1H, 2H, and 3H, were 3.5, 2.9, 1.7, and 0.7 g, respectively, for inoculated plants and 5.6, 4.4, 2.8, and 1.3 g, respectively, for noninoculated plants.

Plants grown at the 0.5H level were chlorotic, and symptoms developed more slowly than at higher levels.

TABLE 3.—*Optical-density assays of tobacco ring spot virus in extracts from cucumber grown at 4 levels of nitrogen*

Days after inoculation	Nitrogen levels (ppm)	Optical density
10	21	0.038
	210	0.205
	630	0.652
	1050	——[a]
13	5	0.100
	21	0.390
	210	0.470
	630	0.750
21	5	0.0 [b]
	21	0.060
	210	0.225
	630	0.760

[a] High nitrogen level caused death of plants.
[b] The optical-density value of the extract from the control plants was equal to that of the extract from infected plants.

TABLE 4.—*Optical-density assays of tobacco ring spot virus in extracts from cucumber grown at 4 levels of phosphorus*

Days after inoculation	Phosphorus levels (ppm)	Optical density
10	3	0.085
	93	0.132
	237	0.350
	547	0.565
18	3	0.300
	93	0.375
	237	0.800
	547	0.650

One week after inoculation, most plants grown at 0.5H showed only a slight systemic mottle, whereas plants grown at 1H and 2H had developed a conspicuous systemic mottle. Systemic mottling was not as severe in plants grown at 3H, although stunting was extreme and plants died in some experiments. Two weeks after inoculation, plants in all treatments showed severe mottling.

The pattern of virus multiplication was not consistent, and differences among treatments were usually small. Virus concentration was always lowest in plants grown at 3H, but maximum virus concentration in different assays was in plants grown at 0.5H, 1H, and 2H. The data suggest, therefore, that multiplication of tobacco ring spot virus in cucumber is not affected greatly by the osmotic concentration of the nutrient solutions in which the host plants are grown.

DISCUSSION. — Sand-culture nutrition experiments have shown that patterns of virus multiplication in relation to nutrition are dependent on specific host-virus complexes (1, 6, 8). Some viruses increase in concentration with increasing nutrient level, whereas others show the same response to nutrition as does the host. The pattern of turnip mosaic virus concentration varied somewhat with time after inoculation (6). Such results have been obtained by using either or both the local-lesion and the spectrophotometric techniques for estimating virus concentration. In the present studies, local-lesion data for virus X were often unsatisfactory, either because differences were too small to be significant or because data were inconsistent. It has been mentioned in another paper (5), that the local-lesion assay used herein for virus X may be less accurate than local-lesion assays for some other viruses and is not accurate enough to measure small differences. The small differences in local-lesion numbers obtained in the assays of plants grown at the higher nutrient levels were not unexpected; they often were associated with little or no differences in symptom expression.

Varying concentrations of nitrogen had a greater effect on symptom expression, as well as on multiplication of both virus X and tobacco ring spot virus, than did variations in phosphorus or in the concentration of a balanced nutrient solution. Concentrations of virus X in *N. tabacum* and in *N. glutinosa* and of

tobacco ring spot virus in cucumber increased with increasing concentration of nitrogen even when the level of nitrogen was sufficient to cause stunting of the host. This pattern of virus multiplication in relation to nitrogen nutrition resembles that reported by Weathers and Pound (8) for tobacco mosaic virus. It differs from the multiplication patterns reported by Cheo, Pound, and Weathers (1) for cucumber mosaic virus in spinach and by Pound and Weathers (6) for turnip mosaic virus in *N. glutinosa*. In these 2 cases, virus concentration increased with increasing nutrient concentration up to 630 ppm, and beyond this both virus multiplication and host growth decreased, i.e., the multiplication patterns of these viruses coincided closely with the growth curve.

Studies of phosphorus in relation to virus multiplication have shown that concentrations of cucumber mosaic, turnip mosaic, and tobacco mosaic viruses (1, 6, 8) increase with increasing concentrations of phosphorus even though levels are high enough to cause stunting. Local-lesion differences in assays on plants grown at 547 and at 237 ppm were significant for cucumber mosaic virus and turnip mosaic virus but not for tobacco mosaic virus. In the present studies on virus X and tobacco ring spot virus, data were not sufficiently consistent to define the exact level of phosphorus that is optimal for virus multiplication. The optimum lay in the range of 237 and 547 ppm. To find out whether virus concentration is more closely associated with host growth or with level of phosphorus, it would be necessary to use a level of phosphorus that is sufficiently toxic to cause consistent stunting of hosts. Concentration of both viruses was reduced at low phosphorus levels.

Both virus X and tobacco ring spot virus showed a wide range of tolerance to concentrations of balanced nutrient solutions. This is in contrast to data obtained for tobacco mosaic virus, turnip mosaic virus, and cucumber mosaic virus (1, 6, 8). Multiplication patterns of these 3 viruses were closely associated with host growth curves.

For experiments in which concentrations of phosphorus and of a balanced nutrient solution were varied, there were small symptom differences among treatments and small growth differences. Some of the variability of data may have been due to the assay method used. Except in these studies and in studies made concurrently on effects of temperature on multiplication of virus X (5), optical-density readings for virus concentration of sap extracts prepared by centrifugation have been made only with tobacco mosaic virus. Work with this virus is facilitated by the facts that it invades tissues rapidly, reaches a high concentration, and is readily purified. Virus X and tobacco ring spot virus are more slowly invasive than tobacco mosaic virus and do not reach such high concentrations. In studies with the viruses used in the present work, it was not possible to purify the sap extracts to the extent that impurities were negligible, and this may have caused variation in the assays. In an attempt to overcome this difficulty, the partially purified virus extracts from infected plants were compared with similarly purified extracts from healthy plants. It is assumed that differences in optical-density readings were proportional to virus nucleoprotein concentration, but it was not possible to prove this.

Differences among individual plants with respect to length of incubation period and susceptibility may be additional factors associated with inconsistencies in the data. For example, cucumber plants receiving the same nutrient treatments showed considerable variability in incubation period following inoculation with tobacco ring spot virus.

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SOME FACTORS AFFECTING THE PURIFICATION OF A STONE FRUIT VIRUS[1]

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SUMMARY

A stone fruit virus that is mechanically transmissible to cucumber has been partially purified by differential centrifugation. The virus is very unstable and is denatured by heat, high salt concentrations, oxidation, and acids. Even in a partially purified state, infectivity is lost in a few weeks at 40°F. The virus has an electrophoretic mobility of 6.7×10^{-5} in a buffer of 0.02 M neutral potassium phosphate plus 0.15 M KCl. Its concentration in cucumber tissue is about 50 ppm of the fresh weight. All attempts to free the virus from impurities have so far failed.

Of approximately 50 stone fruit viruses reported in North America[3], only 1 is known to be mechanically transmissible[4]. None of the stone fruit viruses has been isolated in pure form. We have been concerned particularly with a stone fruit virus, designated PLMV for convenience[5], that is mechanically transmissible to cucumber. The identity of this virus has not been established because it has not been transmitted from infected cucumber back to fruit trees. In this paper we present a procedure developed to purify partially PLMV from cucumber while maintaining a high degree of infectivity.

The virus is readily transmissible to the cotyledons of cucumber seedlings from a number of different fruit tree sources. A culture of this virus has been maintained in Chicago Pickling cucumber by serial transfer at 1- to 2-week intervals for several years. This culture, C-60, was used in all the work reported here.

Homogenates of diseased cucumber tissue, prepared by grinding 1 part of fresh tissue in 3 parts by weight of the buffer described in the next section in a Servall Omni-Mixer for 3 minutes, were used for infectivity studies. The homogenate was kept cool by placing the mixing chamber in an ice bath. The infectivity of the virus was measured by counting the number of starch lesions formed on inoculated cucumber cotyledons following usual local-lesion assay procedures. Details of this inoculation procedure are reported elsewhere[5].

EXTRACTION.—Preliminary studies indicated the virus to be very unstable, and it was necessary to study each step of the purification procedure to avoid denaturation of the virus. Infectivity was lost if homogenates were made in citrate buffer at pH 5, subjected to ammonium sulfate or acetic acid precipitation, or heated to 50°C. Activity was lost also when leaves were frozen or etherized before homogenization.

The relative infectivities of homogenates prepared with neutral phosphate buffer of different concentrations were determined. The optimum buffer concentration appears to be between 0.005 and 0.01 M (Table 1). When the pH of the buffer was varied by mixing different proportions of 0.007 M K_2HPO_4 and 0.007 M KH_2PO_4, the optimum infectivity occurred near pH7.

In neutral phosphate, homogenates remained infective for only about 12 hours at room temperature, suggesting that the virus is sensitive to oxidation. Neutral phosphate that contained a range of concentrations of sodium bisulfite, thiourea, cysteine hydrochloride, and ethylenediamine tetraacetate were prepared to determine the effects of these materials on infectivity. Cysteine-HCl and ethylenediamine tetraacetate were by far the most effective. The number

TABLE 1.—*Relative infectivities of homogenates from PLMV-infected cucumbers prepared in neutral phosphate buffers of different concentrations*

Molarity of buffer	Number of starch lesions on 10 cotyledons
0	7
0.001	12
0.002	36
0.005	40
0.010	40
0.020	28
0.050	5
0.100	0
0.200	0
0.500	0

[1] Accepted for publication June 13, 1955.
Scientific Paper No. 1303, Washington Agricultural Experiment Stations, Pullman, Washington. Project No. 865.
These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the State College of Washington (NR 134-121), and were supported in part by funds provided for biological and medical research by the State of Washington Initiative Measure No. 171.

[2] Division of Plant Pathology, State College of Washington, Tree Fruit Experiment Station, Wenatchee, and Agricultural Research Service, U. S. Department of Agriculture.

[3] U. S. Department of Agriculture. 1951. Virus diseases and other disorders with virus-like symptoms of stone fruits in North America. U. S. Dept. Agr. Handbook No. 10. U. S. Govt. Printing Office, Washington, D. C.

[4] Moore, J. D., J. S. Boyle, and G. W. Keitt. 1948. Mechanical transmission of a virus disease to cucumber from sour cherry. Science 108: 623-624.

[5] Kirkpatrick, H. C., and R. C. Lindner. 1954. A rapid spectrophotometric assay for some plant viruses. Phytopathology 44: 525-529.

of starch lesions per 10 cotyledons was increased from 40 to 105 by the addition of 0.01 M cysteine-HCl, whereas 0.007 M ethylenediamine tetraacetate increased the lesion count to 139. Mixtures of the 2 materials increased the lesion count to 184.

With some plant tissue, the buffering capacity of 0.007 M neutral phosphate was too low and was increased by adding 0.01 M sodium diethylbarbiturate. The extraction buffer that gave the highest infectivity had the following composition: 0.007 M neutral potassium phosphate, 0.01 M sodium diethylbarbiturate, 0.007 M neutral potassium ethylenediamine tetraacetate, and 0.01 M cysteine-HCl. The buffer was adjusted to pH 7 by addition of 1 M KOH, stored at 40°F, and made fresh at weekly intervals. This buffer was used as standard in all succeeding work.

The inclusion of a small amount of detergent (0.0025 per cent) in the buffer increased the lesion count of the homogenate about 50 per cent, but because the detergent decreased the yield of virus on high-speed centrifugation, its use was discontinued.

Homogenization times of 2, 3, 4, or 5 minutes gave preparations differing little in infectivity. A 3-minute grinding period was chosen as standard.

Little difference in total infectivity was noted when ratios of tissue to buffer of 1:1, 1:2, 1:3, or 1:5 were used. A proportion of 1 part of fresh tissue to 3 parts of buffer was chosen as the most convenient.

PARTIAL PURIFICATION BY CENTRIFUGATION.—Freshly harvested leaves were used. A sample of 25 g of leaves was homogenized with 75 ml of cold extraction buffer. The homogenate was readily freed of the coarser cell debris by vacuum filtration through a thin pad of Pyrex glass wool. Subsequent purification was performed by differential centrifugation in a Spinco preparative centrifuge with a No. 40 head. The finer cell debris was removed by centrifugation at 15,000 rpm for 15 minutes (20,360 $\times g$ max.). A clear straw-colored centrifugate usually was obtained. This was centrifugated at 40,000 rpm for 2 hours (144,700 $\times g$ max.). The pellets were dissolved with 2 ml of cold 0.02 M neutral phosphate buffer containing 0.15 M KCl. This buffer was chosen because the potassium concentration approximates that found in plant cytoplasm, and the buffer has a favorable ionic strength for electrophoresis. Solution of the pellets was aided by stirring with a glass rod several times during a 15-minute period. The low- and high-speed centrifugation cycles were repeated. The final pellet was usually clear and colorless, although a trace of chlorophyll was sometimes present. The final pellet was dissolved with 3 ml of buffer and stored in the refrigerator overnight. The following morning the 3-ml samples were centrifuged at 5000 rpm for 15 minutes (2,264 $\times g$ max.). Two samples were prepared in 1 day with this procedure. Infectivity tests on this partially purified material, with appropriate dilutions, showed that about 50 per cent of the original activity of the homogenate was usually retained. Even in this partially purified state, however, PLMV was unstable and could be kept active for only a few weeks at 40°F.

ELECTROPHORESIS.—The partially purified samples were analyzed electrophoretically to determine the relative degree of purity and to determine the electrophoretic mobility of PLMV. A 2-ml cell in a Perkin-Elmer electrophoresis apparatus was used with a current of 20 milliamps at 90 volts. The phosphate plus potassium chloride buffer used in the centrifugation procedure was used also during electrophoresis so that dialysis of the samples was not necessary. Photographs were taken at 20-minute intervals for 100–140 minutes.

Typical electrophoretic patterns of preparations from normal cucumber cotyledons and from cotyledons

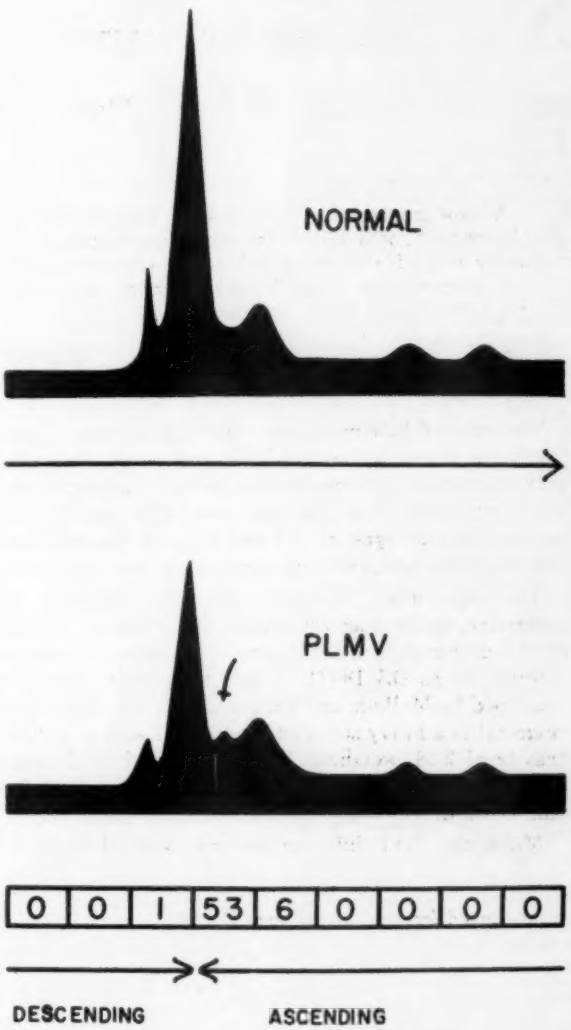

FIG. 1. Sixty-minute ascending electrophoretic patterns of preparations from normal and PLMV-infected cucumber cotyledons. The peak for PLMV is indicated by an arrow. The figures at the bottom indicate the relative infectivities of successive increments of both ascending and descending limbs.

infected with PLMV are shown in Figure 1. Infectivity tests of successive increments of both the ascending and descending limbs of the electrophoresis cell demonstrated that infectivity was associated with the PLMV peak, and typical results of such infectivity tests are also shown in Figure 1. Isolates from cucumber leaves were similar to those from cotyledons except for higher concentrations of normal components.

It was evident that while we had partially purified PLMV, the preparations were still heavily contaminated with normal cytoplasmic constituents of large size. Attempts to effect a better purification of the virus by other cycles of centrifugation or by precipitation with salts, acids, alcohol, or acetone have failed.

The electrophoretic mobility of PLMV was found to be 6.7×10^{-5} in the phosphate plus potassium chloride buffer. The concentration of PLMV in cucumber leaf tissue was calculated to be of the order of magnitude of 50 ppm of the fresh weight. Yields were calculated from the area under the electrophoresis peaks.

TREE FRUIT EXPERIMENT STATION
WENATCHEE, WASHINGTON

SOIL DIFFUSION PATTERN OF 1,2-DIBROMO-3-CHLOROPROPANE [1]

S. T. Ichikawa, J. D. Gilpatrick, and C. W. McBeth

SUMMARY

A new nematocide, 1,2-dibromo-3-chloropropane (Nemagon), was tested for its diffusion pattern in sandy soil. No nematocidal effect appeared when soil samples were taken 1 week after treatment, but the nematocidal effect increased from 2 weeks up to 9 weeks. By the fifth week, the nematocide had spread 13 in. horizontally and vertically and by the ninth week, 15 in. Thus the time elapsing between treatment and sampling is important when evaluating this new fumigant.

The rate of movement and diffusion pattern of soil fumigants through the soil is dependent on the complex interaction of a number of factors. Among these, vapor pressure, chemical reactivity with various soil components, the type of soil and its porosity, moisture content of the soil, and soil temperature are important.

The experiment recorded here was designed to determine, under field conditions, the diffusion pattern of 1,2-dibromo-3-chloropropane (Nemagon, formerly referred to as OS 1897), a new nematocide recently described by McBeth and Bergeson (5). This saturated chemical is a heavy straw-colored liquid with a specific gravity of 2.08, weighing 17.3 lb. per gallon. It boils at 196°C (760 mm Hg) with a vapor pressure of 0.8 mm at 21°C.

METHODS.—Soil diffusion patterns were determined by the method originally described by Thorne (6) and modified by Allen and Raski (2) and by Jensen, Caveness, and Mulvey (3). These modifications included use of a plant parasitic nematode instead of the free-living and saprozoic forms and utilization of an indicator plant instead of the gravity-screening technique for evaluating nematode viability.

The field selected was sandy loam (moisture equivalent 5.9 per cent) heavily infested with root knot nematode (*Meloidogyne* sp.). At the time of injection, the soil moisture was 5.9 per cent of the dry weight, and the soil temperature at the injection point remained at approximately 21°C throughout the experiment, a temperature at which Nemagon is known to show high nematocidal activity. The material was applied 8 in. deep with a tractor applicator equipped with a single chisel. The rate of application of Nemagon was 0.22 ml per linear ft. (2.5 gal. per acre). It was diluted with Shell Special Lacquer

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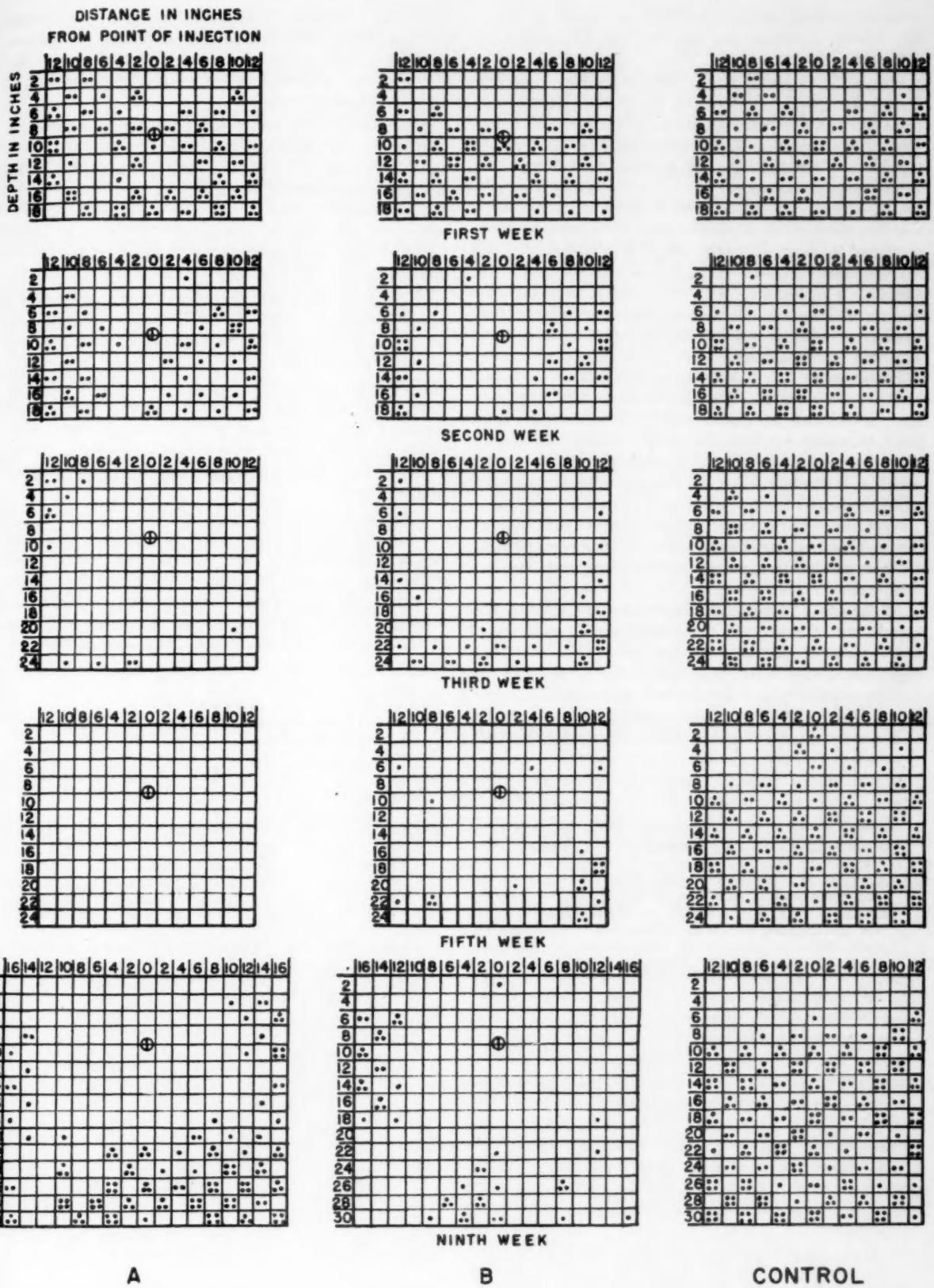
[1] Accepted for publication June 13, 1955.

FIG. 1. Soil diffusion patterns of 1,2-dibromo-3-chloropropane in sandy loam. A and B are replicates. Circled plus signs indicate the point where the nematocide was injected 8 in. deep at the rate of 2.5 gal./acre. Percentages of tomato roots with root knot (plants grown in soil from indicated areas) are indicated by the following: 1 dot, 1–25; 2 dots, 26–50; 3 dots, 51–75; and 4 dots, 76–100.


DISTANCE IN INCHES
FROM POINT OF INJECTION
DEPTH IN INCHES
FIRST WEEK
SECOND WEEK
THIRD WEEK
FIFTH WEEK
NINTH WEEK
A
B
CONTROL

Diluent (boiling range 54–82°C) so that 0.88 ml of the diluted material was applied per linear ft. (10 gal. per acre).

One, 2, 3, 5, and 9 weeks after injection, 2 profiles were taken. Large holes with the line of injection in the center and perpendicular to 1 vertical side were dug. The exposed vertical surface was sampled 13 in. on either side of the injection point to a depth of 18 in. from the soil surface at the 1- and 2-week intervals, and to a depth of 24 in. at the 3- and 5-week intervals. For the ninth week, the width of lateral sampling was extended to 17 in. from the point of injection, and the vertical distance was extended to 30 in. from the surface. The samples were taken in a checkerboard design with a special trowel that cut 2-in. cubes. Control profiles were taken in the same manner from adjacent untreated areas.

Each sample was placed in an individual paper bag and on the same day transferred to a 4-in. clay pot half filled with sterilized soil. Each pot was then sown to tomatoes. Five to 6 weeks after seeding, the plants were harvested and graded according to the number of knots present on the roots, by the method of McBeth and Bergeson (4).

Results.—The results are presented in checkerboard charts that illustrate the diffusion pattern of 1,2-dibromo-3-chloropropane under the conditions of this experiment (Figure 1).

At the end of 1 week, Nemagon had not affected the population of root knot nematodes significantly, even at the point of injection. At the end of 2 weeks, however, nematodes were inactivated for a distance of 5–6 in. vertically and horizontally from the injection point. This zone of nematode inactivation had spread to 9 in. by the third week and to 13 in. by the fifth week. The diffusion pattern after 9 weeks was approximately 15 in. in radius, and although no further samples were taken it seems probable that the chemical had almost reached its maximum sphere of activity by this time.

Discussion.—As compared to other nematocidal soil fumigants, 1,2-dibromo-3-chloropropane diffuses very slowly because of its high boiling point (196°C) and low vapor pressure (0.8 mm at 21°C). Because of the reduced rate of diffusion, the time lapse between treatment and sampling is very important. Samples taken too soon after treatment do not present a true picture of the effective diffusion pattern.

It is known from other tests that Nemagon does not cause immediate death to the nematodes exposed to its vapors. Microscopic observations have shown that as long as 4 weeks after exposure, root knot larvae appear to be unaffected, yet are unable to infect tomato roots. The reason for this is not known. By using the indicator crop technique as described in this paper rather than the funnel or screening technique, a true evaluation of the nematocidal activity of the chemical is determined.

Allen and Raski (1) reported that fumigants gave less control in heavy soil than in light soil and that this was due to their failure to diffuse through soils containing clay or through those high in organic material. Hence, it is possible that diffusion of Nemagon will be less in heavy or organic soil than in the sandy soil in which this experiment was conducted.

Shell Development Company
Agricultural Research Division
Modesto, California

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Yellow-net Virus Disease of some Solanaceous Hosts. R. E. WEBB[1]. During the late summer of 1954 on Aroostook Farm, Presque Isle, Maine, 4 plants of *Physalis floridana* Rydb. that had been used as indicator plants for potato leaf roll virus developed pronounced yellow chlorosis of the vein and veinlets (Fig. 1). Later, 2 of 18 plants of *P. floridana* that had been inoculated with leaf roll virus from diseased potato plants developed similar symptoms. Aphid transmissions from infected plants of *P. floridana* to healthy plants of *P. floridana*, *P. angulata* L., and *Datura stramonium* L. indicated the virus nature of the disease. The virus was not mechanically transmissible.

Symptoms were similar on infected plants of the hosts mentioned previously. The first evidence of infection was noted on the older leaves. Small, bright-yellow, slightly elongated spots appeared on the veins near the leaf apex. Subsequently, the veins and adjacent veinlets became chlorotic, and in 3–7 days the entire veinal network became affected. Most of the mature and maturing leaves on rapidly growing young plants developed vein-netting. As infected plants grew older, fewer of the leaves became affected, and in 2–3 months the veinal chlorosis faded and disappeared from the mildly affected leaves. Infected plants 4 months old and older rarely showed vein-netting except near the apex of an occasional leaf. Diseased plants were only slightly stunted and set fruit normally. Seed from infected plants produced only healthy seedlings.

The average incubation periods of the virus in plants of *P. floridana*, *P. angulata*, and *D. stramonium* were 14, 23, and 27 days, respectively, at a temperature of 24°C.

In addition to *Myzus persicae* (Sulz), the foxglove aphid *Myzus solani* (Kalt.), the buckthorn aphid *Aphis abbreviata* Patch, and the potato aphid *Macrosiphum solanifolii* (Ashm.) were tested in replicated series as possible vectors. Only *M. persicae* transmitted the virus in these tests.

When *P. floridana* was used as a virus source in 183 trials with 10 aphids to each test plant, 63 per cent of the attempted transmissions were successful. The efficiency of transmission from infected plants of *P. angulata* and *D. stramonium* was less. In replicated trials, the minimum virus-acquisition period was 1 hour, and the minimum test feeding period necessary for infection was 2 hours. Once an aphid acquires the virus it may remain infective for several days. When 10 infective aphids from each of 2 test plants were transferred daily to a series of healthy plants, infections were obtained through the third day of a possible 5 days in 1 series and intermittently through the sixth day of a possible 7 days in a second trial.

Host range studies indicated that plants of tomato (*Lycopersicon esculentum* Mill. var. Rutgers and Marglobe) and potato (*Solanum tuberosum* L., var. Katahdin and Saco) were not susceptible in aphid and approach-graft inoculations. When colonized with infective aphids, plants of sugar beet (*Beta vulgaris* L.), broccoli (*Brassica oleracea* var. *botrytis* L.), kale (*B. oleracea* var. *acephala* DC.), rutabaga (*B. napobrassica* Mill.), turnip (*B. rapa* L.), *Nicotiana glutinosa* L., *N. rustica L.*, and *N. tabacum L.* failed to produce symptoms.

Symptoms and vector relations of the yellow-net virus reported in this paper appear to resemble closely those of the sugar beet yellow-net[2] and tomato yellow-net viruses.[3] Comparative tests with these viruses were not made. Several attempts to infect plants of 2 tomato varieties with the yellow-net virus from diseased plants of *P. floridana*, however, were unsuccessful. Inoculation was by aphids and by approach grafts. In 3 separate replicated trials, sugar beet plants inoculated with the vein-netting virus failed to produce symptoms. Additional studies will be necessary to determine the true relation among these viruses which induce a simi-

[1] Plant Pathologist, Horticultural Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

FIG. 1. Leaf of *Physalis floridana* showing chlorosis of the veins and veinlets induced by the yellow-net virus.

[2] Sylvester, E. S. 1948. The yellow-net virus disease of sugar beets. Phytopathology 38: 429-439.

[3] Sylvester, E. S. 1954. Yellow-net virus disease of tomato. Phytopathology 44: 219-220.

lar disease syndrome in plants of different species.—Horticultural Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

A Method for the Production of High-titer Stocks of Bacteriophages of Xanthomonas pruni.[1] L. B. Bernstein and A. Eisenstark. The usual method for the preparation of bacteriophage stocks is to collect filtrates from bacterial broth cultures after phage lysis. In the case of the T-series phages of *Escherichia coli* (Mig.) Castell. & Chalmers, higher titers have been obtained by the recovery of phage from agar petri plates in which confluent lysis of bacterial cells has occurred.[2] With the phage of *Xanthomonas pruni* (E. F. Sm.) Dows., the authors have been unable to get stocks beyond a titer of 9×10^8 phage particles per ml with either of these methods. Modification of the agar-layer technique, however, has yielded phage stocks with at least a 10-fold increase in titer.

The procedure of Swanstrom and Adams[2] was followed, except for modifications to be described. Petri plates were poured with 20 ml of nutrient agar base. After the agar hardened, a surface layer was poured that consisted of 5 ml of a mixture of semisolid agar, host cells, and enough phage to produce about 1000 plaques per plate. After adequate incubation to allow for lysis of cells, the surface layers were removed and placed in a sterile pint Mason jar. Old stocks of phage suspensions were used instead of sterile broth for washing and collecting surface layers, thus excessive dilution of the phage present in the surface layer was avoided. The collected material was blended in an Osterizer for 15 seconds. This step accounted in part for the high final titer, probably because the beating process released phage from agar particles. The mixture was stored overnight at approximately 12°C and then blended again for 15 seconds. Bacteria and the remaining solid particles were removed by low-speed centrifugation. The supernatant fluid was collected and assayed for phage titer.

Table 1.—*Titers of phage obtained by the original and the modified procedures*

Phage	Swanstrom-Adams method	Modified method
Xp1	8.4×10^8	4.5×10^{10}
Xp3	4.6×10^8	8.2×10^{10}
Xp4	2.8×10^8	3.0×10^{11}
Xp8	6.0×10^8	1.2×10^{11}

The blending process was a critical step in the technique. The use of the Osterizer blender for more than 15 seconds, or the use of the more powerful Waring blender, broke up the agar into such fine particles that it was difficult to remove bacteria and agar by centrifugation. Also, excessive blending yielded a lower titer than that obtained by the prescribed method.

Typical examples of phage titers obtained by this modified method are compared in Table 1 with yields obtained with the technique of Swanstrom and Adams.

The relative effect of each modification in the procedure was tested independently to determine the extent to which each influenced the final titer. The use of stock phage suspension for washing and collecting surface layers was found to be responsible for a slight increase in titer, but its primary function was to avoid the dilution effect that would have resulted if nutrient broth were used. The procedure of blending twice with an intermediate incubation was responsible for the further increase in titers.—Department of Bacteriology, Kansas State College, Manhattan, Kansas.

[1] Publication No. 292, Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan. This study was aided by a contract between the Office of Naval Research, Department of the Navy, and Kansas State College, NR 135-172.

[2] Swanstrom, M., and M. H. Adams. 1951. Agar layer method for production of high titer phage stocks. Soc. Exp. Biol. Med. Proc. 78: 372-375.

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The William F. Clapp Memorial Award was presented recently to phytopathologist Carl Hartley in recognition of Dr. Hartley's contributions to the knowledge of wood decay and its control in ships. The award was presented by the Sea Horse Institute, center for research and testing of methods and materials for preventing damage to woods, metals, and other materials in contact with sea water.

Emeritus Professor of Plant Pathology H. W. Anderson will leave San Francisco around October 17 for a "round-the-world" trip. Dr. and Mrs. Anderson expect to visit Japan, the Philippines, Singapore, Indonesia, and Majorca, returning to Urbana, Illinois, by way of New York about June 1, 1956.

Phytopathologist George A. Zentmyer has received the Medal of Honor from the California Avocado Society, for "Outstanding meritorious service in behalf of the California avocado industry."

New Head of the Department of Plant Pathology, University of Idaho, is Arthur Finley. Dr. Finley succeeds Dr. W. C. Hungerford who retired recently.

Plant Pathologist Karl Maramorosch of the Rockefeller Institute spent part of the summer in Mexico on a travel grant from the Rockefeller Foundation. Dr. Maramorosch worked with Dr. John S. Niederhauser of the Mexican Agricultural Program on problems related to virus diseases of corn.

"Results of 1954 Fungicide Tests" reprinted from a series of articles appearing in *Agricultural Chemicals*, April through June, may be purchased in bound and covered form for $1.00 per copy by sending orders with remittance to D. A. Roberts, Department of Plant Pathology, College of Agriculture, Cornell University, Ithaca, New York. The publication of these results is under the sponsorship of the American Phytopathological Society. It is a continuation of the publication of results formerly provided through a Supplement of the Plant Disease Reporter. The Temporary Advisory Committee on Collecting and Disseminating Data on New Fungicide Tests of the American Phytopathological Society arranged for the recent publication of data and the continuation of a program for annual publication of Fungicide Test Results in the future. Dr. D. A. Roberts, Department of Plant Pathology, Cornell University, Ithaca, New York, will be in charge of this project during the current year.

The Northeastern Division will hold its annual winter meeting on November 3 and 4 in the auditorium, Eastern States Farmer's Exchange, Inc., West Springfield, Massachusetts.

The North Central Division held its annual meeting at Wooster, Ohio, August 8-10. About 80 members representing all states of the Division except one were present. The program included exhibits of research in the Department of Botany and Plant Pathology, tours of field plots and station grounds, and a group of short reports on systemic fungicides. A banquet, together with the Ohio Pesticide Institute, was held at Smithville Inn on August 11. Eric Sharvelle addressed the group on the subject "The Role of Chemicals in Agriculture." Officers for the year are C. O. Johnston, President; J. J. Christensen, Vice-President; and M. B. Linn, Secretary-Treasurer. Councilor is J. R. Shay. The 1956 meeting is to be held at Manhattan, Kansas.

Editor: K. W. Kreitlow, Plant Industry Station, Beltsville, Md.

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